ENDURING BEHAVIOURAL EFFECTS IN RATS TREATED WITH CAFFEINE DURING ADOLESCENCE

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Abbreviations

C	Celsius
CNS	Central nervous system
DSM-IV-TR	Diagnostic and Statistical Manual of Mental Disorders (4th Edition, Text Revision)
GABA	γ-amino-butyric acid
GAD	Generalised anxiety disorder
i.p.	Intraperitoneal injection
lx	Lux
mg	Milligrams
mg/kg	Milligrams per kilogram
min	Minutes
ml/kg	Millilitres per kilogram
mm	Millimetres
oz	Ounces
PND	Postnatal days
PTSD	Post-traumatic stress disorder
s	Seconds
S.E.M	Standard error of the mean
SES	Socioeconomic status
US	United States of America

Abstract

Children and adolescents are regular consumers of caffeine, and their consumption is increasing. Caffeine has been shown to affect the later behaviour of rats and mice when exposed to the drug daily before birth and during the lactational period of development. However, to date, little research has investigated the effects caffeine consumption may have on adolescent brain development, and the behavioural consequences of this. The present study, therefore, investigated the effects of repeated caffeine exposure on adolescent rats on behavioural measures of anxiety. During middle and later adulthood, the rats' activity and emotional reactivity were assessed by means of frequencies of rearing, ambulation, immobility, defaecation and urination recorded in an open field, as well as their occupancy of corners and centre squares of the field, and their partial emergence and latencies to fully emerge from a small darkened chamber into a brightly lit arena. The results showed that those rats treated with caffeine were probably more emotionally reactive than untreated controls, as suggested by more immobility, defaecation and urination. There were also effects on rearing and ambulation that might have arisen from increased impulsivity. Overall, the results suggest that exposure to caffeine during adolescence produces some small but significant increases in emotionality in adulthood. This study may have clinical implications, as it is possible that people exposed to caffeine as adolescents, may show increased anxiety later in life.

1.0 Introduction

1.1 General Overview

Not only is caffeine one of the most widely consumed psychoactive drugs (Hughes, 1996; Liguori, Hughes, & Grass, 1997; Nehlig, Daval, & Debry, 1992; Weinberg & Bealer, 2001; Zahn & Rapoport, 1987); people as young as 15 years old begin consuming this substance on a regular basis (Hughes & Hale, 1998). Much is known about the effects of caffeine exposure in human prenatal stages, in neonates, and in adults, but there has been little research undertaken in this area on children and adolescents (Heatherley, Hancock, & Rogers, 2006; Hughes & Hale, 1998). A large US study in 1982 (cited in Hughes & Hale, 1998) surveyed 1135 five- to 18-year-olds, and found that 98% consumed caffeine at least once during the surveyed week. Their average daily intake was 37mg/day (or .9mg/kg/day). A later study found lower caffeine intake than previous estimates and a greater amount of this caffeine was consumed in the form of carbonated drinks (Ellison, Singer, Moore, Nguyen, Garrahie, & Marmor, 1995). As with adults, the large majority of children and adolescents are daily caffeine consumers. However, both total intake and intake corrected for body weight in children are much less than those of adults (Hughes & Hale, 1998). Thus, it is clear that children and adolescents are becoming increasingly exposed to caffeine, but there is little known about the short-term and long-term outcomes of this exposure.

An issue when investigating the effects of caffeine on both humans and animals is the consistency of dosage. In humans, caffeine is generally self-administered and consumed within an everyday diet. Caffeine is found in varying amounts in many different foods and drinks (Hughes, 1996), in which doses can vary widely. This can depend on many factors

such as beverage type, strength of coffee brew, and beverage size. Some general estimates of doses of caffeine are 100mg for 6oz of brewed coffee, 65mg for 6oz of instant coffee, 40mg for 6oz of tea, and 35mg for 12oz of soft drinks (Hughes & Hale, 1998). Considering the caffeine intake of today's adolescents, it is important to appreciate that the main active ingredient in energy drinks is caffeine. The caffeine content in these drinks varies widely, from 50 to 505mg per can or bottle (Reissig, Strain, & Griffiths, 2009). Reissig et al. (2009) expressed concern about energy drinks having a high potential to cause acute caffeine toxicity due to inadequate labelling of caffeine content and the ease of availability for children and adolescents. While energy drinks are not the only source of caffeine for today's adolescents, it is a rapidly growing market (Reissig, et al., 2009).

With the variety of caffeinated products on the market today, and the increasing number of adolescents consuming these products, understanding the effects caffeine may have on this population seems to be important. To do so, understanding the drug and its mechanisms of action is critical.

1.2 Caffeine

Caffeine is a psychostimulant, and one of a group of purine alkaloids (also known as methylated xanthines, methylxanthines, or xanthines, Weinberg & Bealer, 2001). When consumed, caffeine acts as a stimulant influencing the central nervous system (CNS, Nehlig et al., 1992). Caffeine is water soluble and passes easily through cell membranes, including the blood brain barrier, (Weinberg & Bealer, 2001) and the placenta (Lorist & Tops, 2003), allowing it to affect the CNS. The literature clearly demonstrates that no individual cellular mechanism has been found to explain caffeine's neurochemical action. However several

mechanisms have been suggested. Lorist and Tops (2003) have proposed that the CNS effects of caffeine are mediated mainly by its antagonistic actions on adenosine receptors. Methylxanthines produce effects opposite to those of adenosine through non-selectively blocking adenosine receptors, thus reversing the actions of adenosine (Hughes & Hale, 1998).

There are four known adenosine receptors that have been identified: the A_1, A_2 ($_A$ and $_B$) and A_3 receptors. The A_1 and A_2 receptors bind with caffeine at low doses, but the A_{2B} receptor only binds at high doses and the A_3 receptor is insensitive to caffeine (Fredholm, Bättig, Holmén, Nehlig, & Zvartau, 1999). Therefore the A_1 and A_2 receptors are the most important of these receptors in understanding caffeine's mechanism of action on the CNS (Lorist & Tops, 2003). Caffeine's ability to block adenosine effects on these receptors can be observed even at low concentrations, which means its effects can be felt after just a single cup of coffee (Lorist & Tops, 2003). Therefore, studying its relevance to both regular and non-regular caffeine consumers seems critical.

Of the two adenosine receptors, the A_1 receptors are the most prolific in the brain, and are found in the highest densities in the hippocampus, cerebellum and cortex (Fredholm, et al., 1999). Conversely, the A_2 receptors are localised in dopamine-rich areas of the brain, in particular the striatum, nucleus accumbens, olfactory tubercles and extended amygdala (Fredholm, et al., 1999). Adenosine acts pre-synaptically to inhibit the release of neurotransmitters such as acetylcholine, norepinephrine, dopamine, γ amino butyric acid, and serotonin (Hughes & Hale, 1998). Post-synaptically, the adenosine receptors open potassium channels, suppressing neuronal activity (Carlson, 2001).

It is suggested that the anxiogenic effects of caffeine may be due to the antagonism of the A_1 adenosine receptors. It is likely that adenosinergic neuromodulation is involved in increased emotional reactivity in laboratory rodents after perinatal exposure to caffeine for several reasons. Firstly, as discussed earlier, caffeine's acute behavioural effects are most probably due to its competitive antagonism of adenosine A_1 and A_{2A} receptors (Fredholm, et al., 1999) and subsequent facilitation of neurotransmitter activity, especially dopamine (Daly, 1993) and acetylcholine (Carter, O'Connor, Carter, & Ungerstedt, 1995). Secondly, chronic treatment with the drug can up-regulate A_1 receptors in adult and newborn rat brains (Marangos, Boulenger, & Patel, 1984; Saadani-Makki, Frugière, Gros, Gaytan, & Bodineau, 2004). And lastly, rats exposed to caffeine during both gestation and lactation, show heightened sensitivity to acute treatment with adenosine analogues (Fisher & Hughes, 1996). Consequently, increased adenosinergic activity is a likely reason for higher perinatal caffeine-induced emotional reactivity that might be a reflection of greater behavioural inhibition and associated timidity (Reznick, 1999).

Dopamine has been proposed as a mediator in the behavioural effects of caffeine, with the assumption that caffeine indirectly enhances dopaminergic activity through its antagonism of adenosine receptors, in particular the A_2 receptors (Garrett & Griffiths, 1997), which as discussed above, are found in areas where dopamine is plentiful. Garrett and Griffiths (1997) stated that the amphetamine-like profile of caffeine at low doses suggests that dopamine could possibly be involved in the subjective effects experienced after caffeine intake. Dopamine's pharmacology changes during the transitions from pre-weaning, to puberty, to adulthood in the rat. Consequently, the brain may be susceptible to the effects produced by caffeine when administered during adolescence (Andersen, 2003).

1.2.1 Known Effects of Caffeine in Rodents

Caffeine has been shown to affect the later behaviour of rats and mice when exposed to the drug daily before birth (Nehlig & Debry, 1994) and during the lactational period of development either in their mothers' milk (Hughes & Beveridge, 1991) or via subcutaneous injections (File, 1987). A not infrequent result of gestational exposure to caffeine has been lower activity detectable soon after birth (Concannon, Braughler, & Schechter, 1983) and during adulthood for up to at least 6 months after treatment (Hughes & Beveridge, 1991). Similar outcomes have been observed following postnatal exposure during lactation (Concannon, et al., 1983; File, 1987; Hughes & Beveridge, 1991) and during both gestation and lactation combined (Concannon, et al., 1983; Hughes & Beveridge, 1991). While male offspring are more susceptible than females to caffeine treatment during either gestation or lactation (Hughes & Beveridge, 1987, 1991; Hughes & Loader, 1996), this sex difference is not evident when exposed to the drug during both periods sequentially (Fisher & Hughes, 1996; Hughes & Hale, 1998). Decreased activity following gestational and/or lactational exposure to caffeine has been interpreted as a reflection of heightened emotional reactivity or timidity (Hughes & Beveridge, 1987, 1991; Hughes & Loader, 1996). Evidence supporting this view includes increased open-field defaecation (Butcher, Vorhees, & Wooten, 1984; Hughes & Beveridge, 1991), longer latencies to enter a conditioned aversive environment (Sinton, Valatx, & Jouvet, 1981) or to emerge from a darkened chamber into a brightly lit arena (Hughes & Beveridge, 1987, 1991), and greater preferences for a black rather than white environment (File, 1987).

1.2.2 Anxiogenic Effects of Caffeine in Humans

There are differing opinions about the effects caffeine may have on the anxiety levels in humans (Smith, 2002). Only a few studies show increases in anxiety following administration

of caffeine. However, there remains uncertainty about the direction of the relationship between anxiety and caffeine: researchers are as yet, uncertain if caffeine leads to mood problems when the person ingesting it already has a high level of anxiety. It has been suggested that some people abstain from caffeinated drinks because of the associated agitation and anxiety. In children who do not regularly consume caffeine, high doses of the drug can produce unpleasant subjective feelings such as "nervousness, jitterness [sic], stomachache, and nausea" (Hughes & Hale, 1998, p.92).

While the study of the anxiogenic effects of caffeine is relatively recent, it has long been acknowledged that caffeine can cause symptoms of anxiety (Hughes, 1996). In his review, Hughes (1996) stated that consuming caffeine on a regular basis can lead to 'caffeinism'. This presents as edginess, irritability, and tremors, and can be very difficult to differentiate from chronic anxiety (Hughes, 1996) without sufficient investigation of the individual's caffeine intake (Andrews, Creamer, Crino, Hunt, Lampe, & Page, 2003; Greden, 1974). While it is widely believed that many people consume caffeine in order to experience its stimulating effects, some research has raised reservations over this assumption (Stern, Chait, & Johanson, 1989, cited in Hughes, 1996; Hughes & Hale, 1998).

While caffeine may indeed cause experiences of anxiety when consumed, it is also capable of aggravating the anxiogenic effects of situations which are already associated with stress (Hughes, 1996). This phenomenon has been found in animal as well as clinical research (Hughes, 1996). For example, physiological concomitants of anxiety such as plasma renin, hypertension, elevated corticosterone levels and increased adrenal weights in mice were increased by the consumption of caffeine (Henry & Stephens, 1980). Clinically, this is important to consider, as it has been reported that in times of stress, caffeine consumption

may increase. This may be particularly relevant to the tendency for adolescents and young adults to caffeine routinely during stressful periods, such as before school and university examinations (Hughes, 1996).

Much research looking at the anxiety-producing effects of caffeine has used very high doses which may not replicate normal human caffeine intake (Quinlan, Lane, Moore, Aspen, Rycroft, & O'Brien, 2000; Smith, 2002), especially as many studies involve the equivalent of a total daily amount of caffeine in one single dose, when caffeine is typically consumed in smaller doses over a longer period of time (Smith, 2002). Overall, the literature suggests that extremely high does of caffeine may increase acute anxiety, but this is rarely seen within the range of normal human caffeine intake (Smith, 2002). This, however, does not address the long-term anxiogenic effects of caffeine. Long-term drug effects may in fact be the function of a phenomenon known as neuronal imprinting, which posits that the effects of a drug may manifest themselves long after exposure to the drug has finished (Andersen & Navalta, 2004). Therefore, the acute anxiogenic properties of caffeine may be less relevant in this study.

1.2.3 Caffeine and its Interactions with Other Social Drugs

It has been found that caffeine consumers are more likely to be smokers and consume more alcohol (Hewlett, 2006). The finding that alcohol consumption may be increased in caffeine consumers has been attributed to the fact that alcohol is a strong inhibitor of caffeine metabolism (George, Murphy, Roberts, Cooksley, Halliday, & Powell, 2008), which may increase and prolong the positive effects of caffeine intake. Research with adults has found that caffeine dependence may be associated with cigarette smoking (Bernstein, 2002), and that smokers also consume more caffeine than non-smokers (Reissig, et al., 2009). This effect

has been explained, to some extent, by an increase in caffeine metabolism among cigarette smokers (Reissig, et al., 2009). Human and animal studies have found that caffeine increases the reinforcing properties of nicotine (Reissig, et al., 2009), and thus some researchers have expressed concern over caffeine's potential status as a gateway drug (Bernstein, 2002). Bernstein et al. (2002) found that teenagers displaying abuse or dependence of any other drug consumed significantly more caffeine than those with no drug abuse or dependence. The authors go on to question whether early and chronic caffeine use may establish dependence on the drug, and whether it may in fact, facilitate other drug-taking behaviour such as nicotine, marijuana and alcohol.

1.3 Anxiety

The experience of anxiety is a normal human phenomenon. In fact, moderate levels of anxiety can be valuable in improving performance, and even severe anxiety can be perceived as normal in the appropriate situation or context (Andrews, et al., 2003). Anxiety has been described as a sense of uncontrollability, during which the individual is predominantly focused on future threats, danger or upcoming potentially negative events (Barlow, 2000). Once an individual has identified a situation as being a threat, the automatic, physiological response is an increase in arousal, leading to action. If action is not possible, the physiological arousal is experienced as symptoms associated with the fight-flight response, or anxiety (Andrews, et al., 2003). Anxiety symptoms include: heart palpitations, dry mouth, nausea, gastrointestinal discomfort, difficulty breathing, hyperventilation, numbness, dizziness, muscle tension, trembling, and narrowing of attention (Andrews, et al., 2003; Wiedemann, 2001). Lader & Bruce (1986, cited in Hughes, 1996) have stated that the

negative anxiety symptoms produced by caffeine can be very similar to those characterising generalised anxiety.

As anxiety is such a common and costly problem in today's society, understanding its etiology and mechanisms of action seems vital. There are many ways to undertake the assessment of anxiety, such as clinical studies, using individuals already seeking medical or psychological intervention for their anxiety, or through animal models. Animal models of anxiety are based on the assumption that anxiety in animals is comparable to anxiety in humans. While it cannot be verified that an animal experiences anxiety in the same or similar ways to human beings, it has been observed that rodents display distinct behavioural patterns which can indicate anxiety (Ohl, 2003). Some of the methods of assessing anxiety in animals, and a number of the limitations of doing so, are discussed below.

1.3.1 Assessing Anxiety in Rats

Millan (2003) discusses the difficulties in assessing anxiety experimentally. 'Anxiety' or anxious states, are human descriptions for mood states experienced by people, and therefore much inference must take place when undertaking the behavioural study of anxiety in animals. No single operational measure of anxiety exists, however the term 'emotionality' has been used to describe the anxious state experienced by animals (Archer, 1973). In order to study anxiety experimentally through animal models, one must establish some parameters which reflect these anxious states. As animals cannot describe the feelings they are experiencing, it is consequently up to the experimenter to infer, from their behaviour, the mood or emotional state the animal is going through. Some bodily functions can indicate the 'emotions' animal may be experiencing. Tachycardia (increased heart rate), increase in

arterial pressure, and hyperthermia can all indicate anxiety in non-human subjects. However, these are often impractical to measure when using animal behavioural paradigms (Millan, 2003). Behavioural markers in rodents, understandably, look somewhat different to those generally exhibited by humans.

As will be outlined below, several observable behaviours in rats have been considered to be indices of emotionality. An understanding of these terms in the context of animal models of anxiety is beneficial, therefore a brief explanation of some the key behaviours follows:

- *Exploratory behaviour* in rodents encompasses a broad spectrum of behavioural patterns such as risk assessment behaviours, walking, rearing, climbing, sniffing, and manipulating objects (Ohl, 2003).
- *Locomotion* is generally measured as the area covered by the animal in a particular test, for example in the open field. Locomotion scores do not generally describe any other behaviours in which the animal may be engaged, nor do they infer a 'motivation' for the locomotion.
- *Freezing* refers to sudden immobility, which may last for a specified period of time.
- *Defaecation* is generally measured in number of faecal boluses, while *urination* may be measured in volume, or frequency.
- When a rat stands upright on its hind legs, this is referred to as *rearing*. In this study, the frequency of rearing was measured, as well as the location of the rearing.
- *Emergence latencies* refer to the time taken for the subject to emerge from one environment to another (generally more aversive) environment.

As discussed above, behavioural markers must be relied upon to reflect the emotive experiences of the animal. For example avoidance, escape, and freezing all indicate that an animal may be experiencing anxiety, despite the fact that humans do not necessarily display

these behaviours when anxious (Archer, 1973; Millan, 2003). Hall (1934) originally related emotionality in rats to the level of sympathetic nervous activity, selecting urination and defaecation as his primary measures. Many researchers consider exploration or locomotion to be inversely related to emotionality (Archer, 1973). Consequently, a motor response, or lack thereof, such as freezing, can be interpreted by the experimenter as indicating a particular mood state, for example, anxiety (Millan, 2003). Decreased activity following gestational and/or lactational exposure to caffeine has been interpreted as a reflection of heightened emotional reactivity or timidity (Hughes & Beveridge, 1987, 1991; Hughes & Loader, 1996). Evidence supporting this view includes increased open-field defaecation (Butcher, et al., 1984; Hughes & Beveridge, 1991), longer latencies to enter a conditioned aversive environment (Sinton, et al., 1981) or to emerge from a darkened chamber into a brightly lit arena (Hughes & Beveridge, 1987, 1991), and greater preferences for a black rather than white environment (File, 1987). However, interpretation of these behaviours may be less straightforward than commonly thought. For example, Lester (1968, cited in Archer, 1973) referring to data from an enclosed maze, suggested that low and high fear states are associated with low exploration, whereas at intermediate fear states, exploration is high, thus producing a U-shaped curve. Impulsivity, panic response and escape behaviours may also be contributing to faster emergence latencies, high locomotion scores and rearing behaviours (Evenden, 1999; King, 1999a).

Hughes (1996) discussed the anxiogenic effect of caffeine in its clinical and animal domains. As he stated, several studies have proposed that regular consumption of “high doses” (p.37) of caffeine, can be related to high levels of chronic anxiety. It was shown using prenatal exposure to caffeine in rodents, that the drug can have modest effects on the developing brain, which can later have influences on behaviour (Hughes & Beveridge, 1987).

Importantly, these authors reported chronic increases in behaviours which are associated with heightened emotional reactivity. While this study and others (eg. (Hughes & Beveridge, 1990, 1991; Hughes & Loader, 1996) focused on prenatal or lactational exposure, it is also reasonable to expect that the development of the brain during adolescence could be affected after exposure to caffeine.

1.3.2 Open-Field

In the assessment of emotionality and anxiety, the open field is one of the simplest and most popular tests currently in use (Brain & Marrow, 1999). The actual open-field procedure normally consists of forcing the rodent to confront an aversive situation (Belzung, 1999).

> "The test consists of the measurement of behaviours elicited by placing the subject in a novel open space from which escape is prevented by a surrounding wall. The elicitation of these behaviours is dependent upon the interaction of the animal with a variety of test factors such as (a) stimulation as a result of removal from a familiar home environment; (b) stimulation involved in transferring the animal to the open field; (c) exposure to the test environment, consisting of both the open field itself and its surroundings; and (d) all prior experience of the test situation" (Walsh & Cummins, 1976, pp.482-483).

Behaviours elicited by placing the rodent in this novel environment are measured. Different versions of the test are in use, with variations in lighting, shape and the presence of various objects placed in the arena (Belzung, 1999). Originally most open fields were circular, but square and rectangular designs later became common (Walsh & Cummins, 1976).

The open-field paradigm has been long used as a tool for the study of emotionality. Once a rat is placed in the open field, various behavioural measures can be evaluated. Assessment of emotionality can include measuring locomotion, ambulation, rearing, freezing, urination and defaecation. Rearing, locality and quantity of movement, and defaecation, have all been

labelled behavioural measures of emotionality (Archer, 1973; Ivinskis, 1966, 1968, 1970). Early research found that more emotional rats exhibited fewer entries in the central part of the apparatus, and higher levels of defaecation (Hall, 1934).

Rearing is a behaviour which, while easy to measure, is often ambiguous in its interpretation. The experimenter must infer the rearing behaviour's purpose – normally either as evidence of anxiety, through attempting to escape, or as curiosity. One might expect that rearing behaviour at the perimeter of the enclosure may be indicating anxiety and an animal's attempt to escape; whereas rearing behaviour in the inner area may be more indicative of curiosity, namely exploration of the upper parts of the apparatus. The animal's presence in the inner area itself, would normally indicate decreased anxiety. However, Archer (1973) indicated that there is little consistent relationship between centre entries and defaecation in the study of emotionality. Most animals do show active escape behaviour as a response to aversive stimuli, which may account for jumping and rearing behaviours which have been observed, especially close to the walls, and may give support to researchers inferring that these behaviours do indeed indicate emotionality (Archer, 1973).

Ambulation is assessed in relation to either lines drawn on the floor or using photocell beams. As the rodent passes through each beam or crosses a line, one unit of locomotor behaviour is recorded (Brain & Marrow, 1999). Higher counts of locomotion could indicate less anxiety. Also, the number of faecal boluses deposited in the field can be counted as a measure of anxiety, with increased defaecation indicating higher anxiety (Brain & Marrow, 1999). Ivinskis (1966) stated that satisfactory measures of emotionality can be obtained using defaecation scores, and a form of urination scoring. The author found that both correlated significantly, supporting Hall's (1934) original findings. However, the methods of measuring

urination vary. It is possible to count the frequency of urination, but it is difficult to measure the volume of it due to the varying quantities of urine eliminated (Ivinskis, 1966). Ivinskis (1966) proposed a urine weighing method to counter this problem, as his study found that frequency of urination was an unstable measure over long-term testing. There is also a problem in inferring the reason for urination. Body weight, sex and metabolism may all contribute to varying urination levels in subjects (Ivinskis, 1968), all of which are seemingly unrelated to emotionality.

The open field has been long established as an appropriate test for measuring situational anxiety in rodents (Millan, 2003), however, other behavioural tests of emotionality exist. The emergence test measures behaviours in a different setting to the open field, hence providing additional information about emotionality. This test is discussed further below.

1.3.3 Emergence Test

The emergence test is based on rodents' inherent aversion to brightly illuminated areas and on their spontaneous exploratory behaviour response to mild stressors, such as novel environment and light (Bourin & Hascoët, 2003). The emergence test consists of a small dark start box, which opens out into a larger brightly lit chamber. Access to the lit chamber is normally facilitated by the experimenter exposing a gap between the two areas. This then creates a natural conflict situation as the animal is exposed to an unfamiliar environment. The conflict is between the tendency to explore and neophobia (Bourin & Hascoët, 2003). Neophobia is a reaction characterised by a hesitancy to engage with novel objects, places, or conspecifics (Paré, Tejani-Butt, & Kluczynski, 2001). The exploratory activity observed when a rodent is placed in the emergence test reflects the combined result of these tendencies in novel situations (Bourin & Hascoët, 2003).

Behaviours considered to be indicative of emotionality in the emergence test differ somewhat to those measured in the open field. Two recent studies recorded the following behaviours: emergence latency, number of head pokes, and activity (Paré, et al., 2001; Thompson, Li, Clemens, Gurtman, Hunt, Cornish, & McGregor, 2004). Emergence latency is recorded at the time the rodent fully emerges from the start box into the brightly lit chamber. Head pokes rely on a consistent operational definition, as this behaviour can be somewhat ambiguous. Head pokes are generally recorded when the rodent partially emerges from the start box and then retreats. Head pokes could be interpreted as either risk assessment behaviour, with risk assessment indicating general anxiety (Paré, et al., 2001), or as behaviours demonstrating curiosity. Activity was measured by Paré et al. (2001) as the number of squares in the lit chamber traversed with all four feet. These behaviours are easily recordable and measurable, and are a good indication of emotionality and neophobia (Paré, et al., 2001; Thompson, et al., 2004).

1.4 Adolescence

Most caffeine chronically administered to developing rats has taken place either prenatally, or very soon after birth. Until quite recently, animal research has not been concerned with periadolescence as a period during which there could be vulnerability to substance abuse (Smith, 2003). Some researchers have claimed that humans are the only species to undergo the period of adolescence, but as Spear (2000) argues, even adolescent rodents display typical signs of adolescence, such as developmental hyperphagia and accelerated growth rates. As well as these signs, gonadarche, increases in social interactions and risk-taking activity have been exhibited in other animal species during this stage of life, all of which are considered to

be indicators of adolescence in humans (Spear, 2000). Other researchers have developed working definitions for rodent adolescence (Andersen, 2003; Andersen & Navalta, 2004; Smith, 2003). Rats enter late adolescence around post-natal day (PND)45 and this stage lasts until approximately PND55 (Andersen, 2003; Andersen & Navalta, 2004). Smith's definition of rat adolescence using the detection of mature diurnal gonadotropin cycling as the indicator, suggests that dosing to incorporate all of the adolescent period should include PND28-60. Referring to Andersen and Navalta's (2004) study above, administering caffeine to rats between PND45 and 55, comes within Smith's (2003) definition of adolescence.

The trajectory of mammalian brain development occurs in multiple stages. Research shows that the human brain is still developing during the adolescent period, and changes appear to continue into the twenties (Winters, 2004). The nucleus accumbens, amygdala and prefrontal cortex which undergo maturation during childhood (Winters, 2004), as well as late developing structures, including the cortex, hippocampus and the cerebellum (Andersen, 2003), mean that different brain regions appear to be vulnerable at varying periods of development. During the periadolescent period, the brain develops an excess of synapses and receptors, which is then followed by synaptic pruning or competitive elimination. This developmental strategy has been observed in humans, primates, and rats (Andersen, 2003). Steinberg (2005) labels adolescence as a particular period of vulnerability in terms of neural systems, and its connections with behavioural and cognitive systems. He suggests that these connections are either weak or unconnected at this stage of development, especially as these systems mature at different rates. The reorganisation of regulatory systems creates a period of neural development which is particularly sensitive (Steinberg, 2005). During pre-puberty several neurochemical changes also occur. Inappropriate stimulation, such as drug exposure during these phases, can cause abnormal development, whereas appropriate stimulation

during critical periods are necessary for appropriate maturation to occur (Andersen, 2003). Due to the complex structural and neurochemical changes which occur during the periadolescent to adolescent periods, consumption of some drugs during late development may cause different effects from those seen when these drugs are consumed during adulthood (Smith, 2003).

The adolescent CNS is known to remain plastic in response to some types of manipulations. For example, with alcohol, it has been reported that the developing CNS is even more susceptible to alcohol-induced changes during postnatal dendritic elaboration than in neurogenesis (Smith, 2003). As dendritic elaboration continues well into adolescent development, susceptibility to alcohol-induced brain damage may also continue on in later development. Damage to structures such as the frontal cortex, neuronal loss in the cerebellum, basal forebrain and neocortex have all also been demonstrated when the developing brain is exposed to alcohol (Winters, 2004).

Administering caffeine, or any psychotropic drug during adolescence is of interest as the maturation of motor behaviour and mood is fundamentally related to synaptic remodelling or enhanced connectivity, both of which occur before adulthood (Andersen, 2003). Andersen and Navalta (2004) stated that drug exposure during childhood and adolescence alters the development of the areas of the brain which are affected when the drugs are active. There are a number of reports describing subsequent effects of a range of drugs administered to rats and mice during their periadolescent stage of development, however little is known about caffeine in this respect. This is in spite of research showing that a number of other drugs which are popular with human adolescents, such as alcohol, amphetamines and “party pills”, can influence the course of later behavioural development when administered to adolescent rats

(Aitchison & Hughes, 2006; Bergstrom, McDonald, & Smith, 2006; Vorhees, Reed, Morford, Fukumura, Wood, Brown, Skelton, McCrea, Rock, & Williams, 2005). In some cases, such treatment has resulted in the development of higher levels of emotional reactivity, as exemplified by later effects on adolescent rats of a common ingredient of party pills, 1-benzylpiperazine (Aitchison & Hughes, 2006). In view of such outcomes and the fact that the adolescent brain is not fully mature either anatomically or neurochemically (Spear, 2000), it would not be surprising if chronic exposure to caffeine during this vulnerable period were to interfere with normal brain development, and normal, expected developmental pathways could potentially become very different to what would be predicted in normal development. It is surprising then, that the long-term effects of caffeine and other drug exposure on the immature brain have not been sufficiently studied at either the clinical or preclinical stages (Andersen & Navalta, 2004).

1.5 Rationale for Current Study

Perinatal and immediate post-natal caffeine treatment has been shown to result in persistent behavioural changes, such as hyperactivity, extending into adulthood (Tchekalarova, Kubova, & Mares, 2005). Gestational and lactational exposure to caffeine has led to reasonably permanent increased emotional reactivity to testing situations (Hughes & Beveridge, 1991). Evidently caffeine has both acute and long-term effects on emotionality and behaviour in the rat (Hughes, 1996). Recent research has demonstrated that the long-term effects of drug exposure are delayed and may be expressed once the vulnerable system reaches maturation, often during adulthood (Andersen & Navalta, 2004). Despite the prolific research on gestational and perinatal caffeine exposure, little, if any, work has been undertaken

investigating the effect of caffeine administered chronically to adolescent rats, and its long-term effects during adulthood. Likewise, the potentially harmful effects of early drug exposure in humans have received little attention (Andersen, 2005).

2.0 Aims and Hypotheses of this Study

At present, there are no data on the long-term behavioural effects of caffeine administered to rodents during adolescence. This study therefore aimed to investigate the effects of caffeine after administration during adolescence. As so many adolescents consume caffeine on a regular basis, with little known about the long-term effects, the results of this study could provide much-needed information in this area.

This study focused on the long-term anxiogenic effects of caffeine. It was hypothesised that exposure to caffeine during the sensitive period of adolescence may interfere with normal brain development and thus cause long-term increased emotionality in rats. Moreover, it was hypothesised that exposure to a higher dose of caffeine during adolescence would be more effective than a lower dose.

3.0 Methods

3.1 Subjects

The subjects were 27 male and 27 female PVG/C hooded rats chosen from 12 litters, at the breeding colony at the University of Canterbury. All litters were of similar size and contained approximately equal numbers of each sex. The rats were weaned at 30 days of age, caged in groups of 3–4 individuals of the same sex from different litters with free access to food (commercial rat pellets) and drinking water. They were kept in an ambient temperature of 22°C ± 2°C on a 12 hour light/dark cycle (with lights on at 8.00am) .

Procedures for housing, drug treatment and testing of all subjects were approved by the Animal Ethics Committee of the University of Canterbury (see Appendix 1).

3.2. Caffeine treatment

When 45 days old (PND45), the 54 experimental subjects were randomly assigned to a control (0 mg/kg) group, a group treated with 15 mg/kg caffeine or a group treated with 30 mg/kg caffeine. These groups contained equal numbers of each sex and, as near as possible, equal numbers of rats from every litter. Each rat was then given a daily intraperitoneal (i.p) injection (1 ml/kg) of its appropriate dose for 11 consecutive days. Control animals were administered isotonic saline, and caffeine-treated rats received caffeine dissolved in saline. Although it is recognised that humans consume caffeine orally, the i.p route of administration was chosen for ease of delivery.

3.3 Behavioural testing

All rats were run through three open-field followed by three emergence tests between PND72 and PND82 (early adulthood), and this process was repeated again between PND112 and PND122. There was an interval of three days between each pair of tests at each testing age. Exactly 40 days intervened between individual subject's set of tests at the younger and the older age. All testing occurred during the light phase of their light/dark cycle in order to minimise disruption caused by the rats being brought out of a darkened holding room into the illuminated research room.

3.4 Open field Test

The apparatus used was a 600×600 mm wooden open field with walls 250 mm high. It was painted black and the floor was divided into 16 squares by a grid of intersecting painted white lines. The open field sat on a 700-mm high table and was illuminated by overhead fluorescent lighting at 47 lx. An infrared video camera was mounted 850 mm above the floor of the apparatus, and all behaviour of each individual rat was recorded for 5 min after having been placed in the centre of the open field. The rat was then removed and the number of faecal boluses it left in the apparatus (defaecation) and the number of times it had urinated (urination) were counted before the field was washed with a 2% solution of Powerquat Blue disinfectant. The video tapes were later viewed and the following forms of behaviour recorded for each rat:

(1) the number of times it reared up on its hind legs (rearing)

(2) the number of lines crossed by its hind legs (ambulation)

(3) the number of times it remained completely immobile for more than 3 s (immobility)

(4) the number of 3 second observations (signalled by an auditory timer and earphone) in which it was occupying one of the four corners (corner occupancy) or four centre squares of the apparatus (centre occupancy).

3.5 Emergence tests

After each rat completed its time in the open-field test, it was returned to its home cage for several minutes and was then placed in a small darkened chamber in order to measure its speed of emergence into a larger brightly lit arena. The apparatus comprised a 200×150×200-mm-high black-painted wooden box that opened by means of a sliding door into a 500×400×200-mm-high arena with a translucent Perspex floor that was illuminated from underneath by two 16-w fluorescent tubes. The light level in the arena was 172 lx, and it was covered by a wire-mesh lid. The apparatus sat on a 700-mm high table in the same room as the open field. An emergence test consisted of placing a rat in the darkened chamber and, approximately 10 s later, opening the sliding door to allow it access to the brightly lit arena. The number of times it partially emerged was counted (head pokes), and the time it took to fully emerge was recorded by a hand-held stop watch. If it had not fully emerged after 5 min, the trial was terminated and an emergence latency of 300 s recorded.

4.0 Statistical Analyses

Two male control rats died in between the first and second series of open-field and emergence testing at PND72–82 and PND112–122. Therefore, treatment (3)×sex (2)×testing age (2) ANOVAs were performed on all measures recorded in the open field (except urination) and emergence apparatus for the remaining 52 rats that completed testing at both ages. As it was clear that there were no consistent patterns of change for any group on any measure between the three tests conducted at each testing age, the ANOVAs were carried out on individual rats' averages of the three. When significant caffeine treatment effects occurred, post hoc comparisons were made between all individual groups by means of Neumann–Keuls tests. Because of large numbers of 0 scores and thus highly skewed distributions, urination was subjected to nonparametric median tests (Siegel, 1956) to assess the effects of adolescent caffeine treatment and sex on numbers of rats that were not seen to ever urinate. An overall comparison of urination at the two testing ages was made by means of a sign test (Siegel, 1956).

5.0 Results[1]

5.1. Open-field and emergence tests at postnatal days 72–82 and 112–122

5.1.1. Adolescent caffeine treatment effects

Mean ± S.E.M scores following adolescent caffeine treatment for all measures recorded in the open field (except urination) and emergence apparatus during PND72–82 and PND112–122 combined can be seen in Table 1.

Table 1: Mean (± S.E.M.) values of each open-field and emergence test measure (except urination) for both sexes and testing ages combined following adolescent caffeine treatment, and results of ANOVAs.

	Caffeine treatment dose (mg/kg)				
	0 (n = 16)	15 (n = 18)	30 (n = 18)	$F(2, 46)$	p
Rearing	34.50 (± 2.73)[a]	28.47 (± 1.86)[a,b]	35.34 (± 1.68)[b]	3.40	.042
Ambulation	73.76 (± 5.04)	64.79 (± 4.67)[a]	79.32 (± 5.78)[a]	3.74	.031
Immobility*	0.96 (± 0.12)[a]	1.46 (± 0.12)[b]	2.08 (± 0.43)[a,b]	10.74	<.0001
Corner occupancy	50.34 (± 1.75)	54.32 (± 2.62)	53.24 (± 1.78)	0.76	>.4
Centre occupancy	5.25 (± 0.34)	3.89 (± 0.51)	5.07 (± 0.56)	2.30	>.1
Defaecation	0.51 (± 0.16)[a]	1.07 (± 0.37)	1.87 (± 0.43)[a]	3.45	.04
Emergence (s)	110.31 (± 18.84)	143.03 (± 18.06)[a]	66.87 (± 16.51)[a]	4.47	.017
Head pokes	4.40 (± 0.51)[a]	6.41 (± 0.68)[b]	3.44 (± 0.41)[a,b]	7.51	.002

[a,b] Difference between the two groups with superscripts in common significant, $p<.05$, Neumann-Keuls test. *Caffeine treatment x testing age interaction significant (see text).

[1] Further explanation of the results of this study are outlined in the recently published paper: Anderson, N.L. & Hughes, R.N. (2008). Increased emotional reactivity in rats following exposure to caffeine during adolescence. *Neurotoxicology and Teratology*, *30*, 195–201.

Significant caffeine treatment effects occurred for all measures except occupancy of corners or centre squares of the open field. Rats that had been treated with 15 mg/kg caffeine exhibited less rearing than those in either other group and less ambulation than rats treated with 30 mg/kg caffeine. These latter rats were also more immobile than those in the other groups, defaecated more than control animals, emerged faster from the darkened chamber than rats treated with 15 mg/kg caffeine and made fewer head pokes than those in either other group. (A significant interaction occurred between adolescent caffeine treatment and testing age for immobility, $F(2, 46)=4.30$, $p=.019$, and will be described in the next section (5.1.2) dealing with sex and testing age differences.). The numbers of rats that did not urinate in the open field on any of their six opportunities following adolescent treatment with 0 (n=16), 15 (n=18) and 30 mg/kg (n=18) caffeine respectively were 5 (31.25%), 3 (16.67%) and 4 (22.22%). Differences between these groups were not significant, $\chi 2(2)=3.20$, $p>.2$.

Table 2: Mean (± S.E.M.) values of each open-field and emergence test measure (except urination) for males (n = 25) and females (n = 27) and for postnatal days 72-82 and 112-122 (adolescent caffeine treatment groups combined), and results of ANOVAs.

	Sex				Postnatal days			
	Males	Females	$F(2, 46)$	p	72-82	112-122	$F(1, 46)$	p
Rearing	29.31 (± 1.69)	36.85 (± 1.66)	8.64	.005	31.05 (± 1.40)	35.11 (± 1.50)	4.39	.042
Ambulation*	57.08 (± 3.24)	86.93 (± 3.17)	47.22	<.0001	72.69 (± 2.95)	71.32 (± 3.46)	0.18	>.6
Immobility**	1.68 (± 0.19)	1.38 (± 0.14)	2.07	>.1	1.83 (± 0.17)	1.23 (± 0.11)	17.72	<.0001
Corner occupancy*	54.65 (± 1.95)	50.93 (± 1.43)	2.21	>.1	54.33 (± 1.18)	51.25 (± 1.57)	5.76	.021
Centre occupancy*	4.30 (± 0.40)	5.10 (± 0.41)	1.79	>.1	4.58 (± 0.31)	4.82 (± 0.38)	0.34	>.5
Defaecation*	1.53 (± 0.31)	0.85 (± 0.29)	2.37	>.1	1.39 (± 0.26)	0.99 (± 0.21)	4.88	.032
Emergence (s)	120.24 (± 18.02)	93.96 (± 12.92)	1.64	>.2	96.24 (± 11.94)	117.96 (± 11.87)	4.59	.038
Head pokes	4.84 (± 0.46)	4.69 (± 0.53)	0.05	>.8	4.44 (± 0.36)	5.09 (± 0.44)	2.66	>.1

*Sex x testing age interaction significant (see text). **Caffeine treatment x testing age interaction significant (see text).

5.1.2 Differences between the two sexes and testing ages

As can be seen in Table 2, female rats showed significantly higher overall frequencies of rearing and ambulation in the open field than males. More rearing accompanied by less immobility, corner occupancy and defaecation, and longer emergence latencies occurred at the older testing age than when the rats were younger.

However, there were also significant interactions between sex and testing age in ambulation, $F(1,46)=6.06$, $p=.018$, corner occupancy, $F(1,46)=10.32$, $p=.002$, centre occupancy, $F(1,46)=5.46$, $p=.024$, and defaecation, $F(1,46)=8.06$, $p=.007$, along with the significant caffeine × testing age interaction for immobility referred to in the previous section. The sex × testing age interactions are outlined in Table 3.

These revealed significantly more ambulation for females than for males at both testing ages, and a significant decrease in the response from PND72–82 to PND112–122 for males but not for females. While females occupied open-field corners less and centre squares more than males at the older testing age, the two sexes did not significantly differ on these measures at the younger age. Females also occupied corners less and centre squares more at the older than at the younger age, but this was not so for males. Males defaecated more than females at the younger but not the older testing age, and, contrary to females, defaecated less at the older than at the younger age.

The caffeine × testing age interaction for immobility arose from, a significant decrease between days 72–82 and 112–122 only for rats treated with 30 mg/kg caffeine i.e., 2.69 (±0.33) and 1.48 (±0.19) respectively. There were no significant changes between the two

testing ages for either the control, $F(1,46)=2.44$, $p>.1$, or 15 mg/kg caffeine-treated groups, $F(1,46)=0.01$, $p>.9$, and the caffeine treatment effect remained significant at testing ages 72–82, $F(2,46)=10.84$, pb.001, and 112–122, $F(2,46)=4.17$, $p=.022$. Numbers of males (n=25) and females (n=27) that did not urinate in the open field on any occasion were 4 (16.00%) and 8 (29.63%) respectively. This sex difference was not significant, $\chi 2(1)=1.36$, $p>.2$. For both sexes combined, 13 rats urinated less, 17 urinated more and 22 showed no change between PND72–82 and PND112–122. These numbers did not differ significantly, $z=0.55$, $p>.5$.

Table 3: Mean (± S.E.M.) values of four open-field measures for males (n = 25) and females (n = 27) recorded at postnatal days 72-82 and 112-122 (adolescent caffeine treatment groups combined) for which sex x testing age interactions were significant

	Males		Females	
	Postnatal days 72-82	Postnatal days 112-122	Postnatal days 72-82	Postnatal days 112-122
Ambulation	59.27 (± 3.36)[a,c]	54.89 (± 3.70)[b,c]	85.11 (± 3.30)[a,d]	88.74 (± 3.51)[b,d]
Corner occupancy	54.09 (± 1.62)[e,g]	55.21 (± 2.67)[f,g]	54.06 (± 1.75)[e,h]	47.31 (± 1.43)[f,h]
Centre occupancy	4.63 (± 0.39)[i,k]	3.97 (± 0.52)[j,k]	4.54 (± 0.48)[i,l]	5.65 (± 0.51)[j,l]
Defaecation	2.04 (± 0.40)[m,o]	1.01 (± 0.31)[n,o]	0.79 (± 0.31)[m,p]	0.91 (± 0.30)[n,p]

[a-p]Values of $F(1,46)$ and probability levels for comparisons between groups with superscripts in common, [a]32.12, $p < .0001$, [b]48.92, $p < .0001$, [c]4.05, $p = .05$, [d]2.16, $p > .1$, [e]0.04, $p > .8$, [f]6.46, $p = .014$, [g]0.32, $p > .5$, [h]16.49, $p < .0001$, [i]0.06, $p > .8$, [j]4.91, $p = .032$, [k]1.49, $p > .2$, [l]4.46, $p = .04$, [m]6.06, $p = 0.018$, [n]0.03, $p > .8$, [o]12.36, $p < .001$, [p]0.21, $p > .6$.

6.0 Discussion of Results

Clearly, exposure of the rats to caffeine during adolescence resulted in a number of significant outcomes that would at least justify further research and perhaps question the wisdom of consumption of high doses of caffeine by human adolescents. This is because treatment with the drug increased immobility and defaecation in the open field thereby suggesting that it had produced small but long-lasting increases in emotional reactivity (Angelucci, Césario, Hiroi, Rosalen, & Cunha, 2002).

The caffeine treatment also had some complicated effects on ambulation and rearing in the open field i.e., the lowest frequencies of each were associated with exposure to 15 mg/kg (low dose group) while there was no significant difference between 0 mg/kg (control group) and 30 mg/kg (high dose group). Since both measures are believed to be negatively related to emotionality (Archer, 1973), this would suggest that 15 mg/kg had increased emotional reactivity but that 30 mg/kg was paradoxically ineffective in this respect. However, it is possible that while the lower of the two caffeine doses may have indeed increased emotional reactivity and thus interfered with any curiosity-related basis for the two responses, the higher dose may have similarly increased emotional reactivity and suppressed curiosity but may also have initiated fear-induced attempts to escape from the apparatus. This was supported in particular for rearing activity by casual observations that this response seemed to reflect either a more relaxed curiosity-related "interest" in the upper parts of the apparatus, or rather "frenetic" behaviour that gave the appearance of attempts to escape. Obviously further research is required to establish whether or not it is possible to distinguish between two types of ambulation and rearing in terms of their specific motivational substrates.

The finding that the shortest emergence latencies in the emergence apparatus occurred with rats exposed to 30 mg/kg caffeine would also appear to be contrary to the possibility that the drug had increased emotional reactivity. On the other hand, the fact that the lowest frequencies of partial emergence in the form of head pokes also occurred with this group, might be interpreted as being due to increased emotional reactivity. However, this seems improbable because of a positive Pearson correlation between the two responses for all rats combined i.e., $r=0.76$, $p(50)<.001$. Instead, as supported by casual observations, it seems more likely that both responses reflected an increase in impulsivity that has been shown to follow acute caffeine administration in rats (Flora & Dietze, 1993).

Sex differences favouring females in open-field rearing and ambulation for PND72–82 and PND112–122 combined were consistent with the view that females are more active than males (Archer, 1973). There were also some other sex differences that were dependent on the age at which the rats were tested namely, less corner and more centre squares occupancy for females than for males when the rats were tested at PND112–122, but no sex differences in these measures at PND72–82. On the other hand, males defaecated more than females when tested at the younger age, but not when older. These sex differences were consistent with the view that male rats are more emotionally reactive than females (Belzung, 1999; Gray, 1971).

Increases in rearing and emergence latencies for all rats between the two testing ages suggests that, in line with earlier conclusions (Bessa, Oliveira, Cerqueira, Almeida, & Sousa, 2005; Imhof, Coelho, Schmitt, Morato, & Carobrez, 1993), they may have become more emotionally reactive as they grew older. This is because they were slower to emerge from the darkened chamber of the emergence apparatus and engaged in more possibly escape-related

rearing at the later testing age. However, a contrary interpretation would follow the observations that, for males only, ambulation and defaecation declined between the two ages, whereas corner occupancy declined and centre squares occupancy increased for females alone. In addition, immobility also declined only for rats that had been treated with 30 mg/kg caffeine. So, while some of the changes suggest an increase in emotional reactivity with age, namely rearing (possibly) and emergence latencies, others suggest a decrease as reported earlier (Candland & Campbell, 1962; Hughes, 1968; Williams, Carr, & Peterson, 1966) i.e., sex-dependent ambulation (possibly), corner and centre squares occupancy, defaecation and caffeine treatment-related immobility. Clearly, the behavioural processes underlying these different age-related changes can not be conclusively identified without further research.

The effects of treatment with caffeine during adolescence on later immobility, defaecation, and perhaps rearing and ambulation suggest heightened emotional reactivity in a similar manner to that concluded for the subsequent effects of perinatal exposure to the drug (Hughes & Beveridge, 1987, 1991). It is possible that caffeine-treated rats' adolescent experience also increased impulsivity (Flora & Dietze, 1993), as suggested by their emergence latencies and number of head pokes in the emergence apparatus. Overall, it seems likely that the results of the study were due to caffeine effects on adolescent brain development possibly involving adenosine-facilitated increases in neurotransmitter activity (Fredholm, et al., 1999), especially dopamine (Daly, 1993), comparable to what probably characterises pre- and early postnatal development. In addition to having possible implications for the risks of caffeine consumption by human adolescents, the results highlight the need in future research to determine how critical adolescence really is in this respect, compared to other ages that are not commonly regarded as important identifiable stages of brain development.

6.1 Methodological Limitations

There are some methodological limitations to consider before generalising these results. Firstly, it is important to note that the rats were administered caffeine via i.p injection. This poses a number of different problems. Humans tend to self-administer caffeine, and do so orally. This study initially attempted to orally administer rats caffeine, but practically, this proved difficult. Adding caffeine to a cage's drinking water created problems. When unadulterated drinking water was measured to create baseline data, different cages consumed varying volumes of water. Due to the fact that rats were housed in cages of three, it was not possible to measure how much each individual rat consumed. If rats had been housed individually this may have been easier to control, however, practically this was not possible.

It has been suggested that the procedure of i.p. injections alone can have an anxiogenic effect in mice (Lapin, 1995), which potentially makes the procedure less than ideal. However, all groups, treatment and controls, received their appropriate injection solutions in the same manner. Therefore, despite the administration route being imperfect, the effects seen can definitely be attributed to the drug and not to differences in administration route.

This study investigated caffeine consumption purely during the period of late adolescence in the rat. It is likely that human consumption of caffeine occurs throughout the lifetime, with consumption possibly beginning in some children, before the age of five (Frary, Johnson, & Wang, 2005). Therefore the period of time during which caffeine was administered is very selective and short compared to human patterns of consumption of caffeine. However, this study clearly demonstrates the effects caffeine consumption can have in even a very short space of time, which illustrates the clinical importance of the findings – that this age period is potentially vulnerable to insult and drug use.

6.2 Methodological Strengths

There are advantages to using rats when studying drug effects on behaviour. As these animals develop rapidly (Andersen, 2003; Clancy, Finlay, Darlington, & Anand, 2007), the periods of adolescence and adulthood occur much more quickly than they do in adulthood . This means that the time taken for drug administration and testing is much less than if initial research was to be completed on humans.

Although this study aimed to increase awareness about the long-term effects caffeine may have on humans, animal subjects were used to predict human responses to the drug. Therefore some caution must be exercised when generalising these results directly to humans. However, animals can indeed be used to answer questions about behavioural dysfunctions, their underlying neural mechanisms and drugs' effects on behaviour (van der Staay, 2006). In addition to this, animal studies can be useful for assessing the behavioural impact of drugs which may affect human anxiety levels (Ohl, 2003).

While this study did not exactly mimic the human consumption patterns of caffeine, it has described important aspects of the effects of caffeine exposure during adolescence. From the results, we can begin to identify the effects that caffeine may have during adolescent brain development. The amount of brain development that occurs throughout childhood and adolescence is immense, and the potential for long-lasting behavioural effects is evident. This study can therefore be seen as important for initiating and guiding future research into this freely available and widely used drug.

7.0 General Discussion

The results of this study supported the general hypotheses of this research. Treatment with caffeine during adolescence did result in some small but nevertheless significant, long-term increases in emotionality. However, there were some complicated findings which show that our understanding of this area is far from complete.

7.1 Availability of Caffeinated Products

Energy drinks, soft drinks, coffee and tea, are readily available, consumed regularly and often in high quantities by adolescents and young people today. All of these beverages contain varying levels of caffeine. As a consequence, the percentage of young people consuming caffeine today is not only large, but it is increasing (Carlezon Jr. & Konradi, 2004). One study, which examined two-day averages of caffeine intake, found that 91% of males aged 12 to 17, and 88% of females in the same age range consume caffeine (Frary, et al., 2005). The effects of repeated exposure to caffeine in young people without awareness of dosage is surely worth investigating. There are few, if any, psychotropic drugs other than caffeine, which society makes available in such an unregulated (Reissig, et al., 2009) and unlimited manner.

The research of Reissig et al. (2009) into currently available energy drinks shows that their caffeine content ranges from 50mg to 505mg per can or bottle. Hughes (1996) listed caffeine contents for other beverages including ground coffee and coca cola which, in large cups, ranged from 3mg (for decaffeinated coffee) to 128mg (for ground coffee). The caffeine content of energy drinks appears to be significantly higher than these other beverages,

begging the question of why these drinks are allowed to be so heavily marketed towards young people, and why there are no regulations on the selling or labelling of these drinks.

There is a well-established relationship between dietary adequacy and socioeconomic status (SES), which has been documented in the literature. SES is related to factors such as income, education level, occupation, and family size. One particular study has investigated the relationship between family SES and the dietary patterns, particularly caffeine intake, of children aged 24 to 36 months. Significantly higher caffeine consumption was found in the lower SES group at all three stages of the study (Skinner, Carruth, Houck, Morris, Moran, & Coletta, 2000). The researchers found that soft drinks, such as cola beverages, coffee and tea were the main sources of dietary caffeine in these children. Findings such as these suggest that certain sections of society may be at greater risk of adverse effects from caffeine as the consumption of caffeine is higher in these groups. Education or prevention may initially need to be aimed at these at-risk areas. Education can be provided through parents, dietician services (Skinner, et al., 2000), education providers, and through public awareness (Knight, Knight, Mitchell, & Zepp, 2004).

7. 2 Adolescence

As has been discussed, little is known about how caffeine can affect neuronal, structural and neurochemical development in the maturing adolescent brain. However, what *is* known is that during other periods of development, such as during gestation and lactation, caffeine exposure can result in increased emotionality and timidity, both in the short and long-term (Hughes & Beveridge, 1987, 1991; Hughes & Loader, 1996). The adolescent brain is still undergoing massive transformations and development (Andersen, 2003; Steinberg, 2005;

Winters, 2004), and so one must question then, why caffeine, a drug so prolific and common in modern society, is so under-researched in this vulnerable age group.

As rats in this study were administered caffeine only during the period of late adolescence (PND45-PND55), one can infer that the behavioural effects seen were due to the drug's effects on brain development during this time. Many areas of the brain are maturing during this period, including the nucleus accumbens, amygdala, prefrontal cortex, hippocampus and cerebellum (Andersen, 2003; Winters, 2004). With so much development occurring, the vulnerability of the brain to drug effects during this developmental period is clear. It is also known that the effects of drugs on the developing brain may not be known immediately (Andersen & Navalta, 2004). Therefore, investigating the enduring effects of caffeine on the adolescent brain, is important, because it may give a truer picture of what the consequences of such drug use could be. The concept of drug effects lasting longer than the drug exposure itself, is known an neuronal imprinting (Andersen & Navalta, 2004). It appears that the results of this study support the notion of neuronal imprinting. As has been discussed, exactly where in the brain this neuronal imprinting has occurred is not yet fully understood, as caffeine's mechanisms of action are not simple.

The implications neuronal imprinting have for drug taking behaviour during adolescence are fairly clear. Teenagers are generally unaware of the rapidly occurring changes in their brain at the time of drug-taking, and are equally unaware of the potential long-term effects this drug-taking may have. When a sanctioned drug such as caffeine is consumed, there is likely to be little consideration of the possible long-term consequences this may have for behaviour or mood. As caffeine is so prevalent in our diet, and is often not labelled in our foods and drinks (Reissig, et al., 2009), it is even possible that adolescents today are consuming caffeine

when they are not even aware of it, or are not aware of the quantity of caffeine they may be consuming on a regular basis.

7.3 Anxiety

Emotionality, or emotional reactivity, in the rat is regarded as the animal equivalent to human anxiety. This study found small long-term increases in some measures of emotionality in rats exposed to caffeine in adolescence. While more animal research would further increase our understanding of precisely what might be occurring after adolescent caffeine exposure, there is also the need for clinical research into the long-term effects caffeine may already be having on humans. Already, anxiety and anxiety disorders are extremely prevalent in modern, Western society. Anxiety disorders, as a group, are the most common psychiatric disorder in the US (Beidel & Stipelman, 2007), with lifetime prevalence for the group of disorders estimated to be as high as 25% (Hettema, Neale, & Kendler, 2001). The acute anxiogenic effects of caffeine in humans have been established (Hughes, 1996), as have longer term effects in animals when exposed to the drug early in life (Hughes & Beveridge, 1987, 1991).

Many neurotransmitters have been implicated in the initiation and inhibition of anxiety and anxious states, including monoamines, γ-amino-butyric acid (GABA) glutamate, adenosine, cannabinoids, multiple neuropeptides, hormones, and neurotrophins (Millan, 2003). Likewise, several brain structures seem to be involved in the expression of anxiety. The limbic system, and in particular, the amygdala, is associated with anxiety (King, 1999b; Lezak, Howieson, & Loring, 2004). As noted above, the amygdala is maturing and changing during the childhood and adolescent periods (Winters, 2004), which might help explain the anxiogenic effects seen in this study. While there are commonalities in the neurobiology of

anxiety, the varying anxiety disorders appear to be have some associations with different brain structures and chemical pathways. For example, dopaminergic functioning has been found to play a central role in social phobia (Stein, 1998). Serotonin and norepinephrine among other neurotransmitters, have been implicated in panic disorder (Coplan & Lydiard, 1998), and GABA seems to be associated with GAD (Connor & Davidson, 1998). Because of the complicated neurobiology of anxiety, it is difficult to understand completely where exactly caffeine may be exerting its influence. As this study only involved the behavioural effects of adolescent exposure to caffeine, it did not endeavour to investigate the specific brain areas or neurochemicals affected by the caffeine administration. We can infer, however, that the adenosinergic pathways as well as dopaminergic already discussed in this paper are likely to have been affected.

7. 4 Impulsivity

Not only was emotional reactivity increased in rats exposed to caffeine during adolescence in this study, but impulsivity was possibly heightened as well. The idea that impulsivity could be affected by caffeine exposure had not previously been considered, and is yet to be researched in a structured manner. Nevertheless, these behavioural effects are extremely important to consider when reflecting on the implications of this research. The DSM-IV-TR stated that impulsivity can manifest itself in many ways, including impatience and difficulty delaying responses (APA, 2000). Elsewhere, impulsivity has been described as behaviours which are poorly thought out, prematurely expressed, unnecessarily risky, or situationally inappropriate, and which have the propensity to result in undesirable outcomes (Evenden, 1999).

Adolescents are already more likely to engage in risk-taking behaviours than other age groups, and it has been hypothesised that this may have a range of effects, from assisting them to gain the necessary skills to allow them to survive without their parents, to predisposing some teenagers to the use of alcohol or other drugs (Spear, 2000). Increases in impulsivity in this already vulnerable population may result in even more risky behaviours. Evenden (1999) believes that there is no one biological basis for impulsivity, and that there are consequently varying manifestations of this behaviour. As a consequence, researching this area of behaviour may prove somewhat challenging. Nevertheless, based on the results of this study, it seems possible that impulsivity may also be a long-term effect of adolescent caffeine consumption, and thus warrants further research.

7.5 Escape Behaviours

In many species, life-threatening events can set in motion a series of primitive behavioural and autonomic responses which include freezing, increased heart rate, and endocrine changes which prepare the person or animal for a fight-or-flight response. Humans suffering from some of the more severe anxiety disorders may experience recurrent unexpected panic attacks which may be manifested as persistent symptoms of physiological arousal, including hypervigilance, exaggerated startle responses, and accelerated heart rate symptoms, all of which suggest the presence of the fight-or-flight state (King, 1999a).

Some of the behaviours observed in the subjects in this study appear to have been driven by the rat's desire to escape from the apparatus. In particular, some of the rearing and locomotion behaviours in the open field apparatus among the high dose rats looked qualitatively different to other rearing and locomotion behaviours in the other two groups.

Some behaviours began as apparently curiosity-motivated rearing behaviour but then changed into attempts to climb the open field walls, which may have been more indicative of active attempts to escape.

Several behavioural tests have been developed to measure escape behaviours in animals, an example of which can be seen when rats are observed in an elevated plus maze. The behaviours they exhibit in this apparatus are believed to replicate phobic anxiety states and GAD in humans. Whilst this and other tests may successfully replicate several types of human anxiety disorders, few behavioural tests have, as yet, been devised which capture the "chronically hyper-aroused, highly avoidant, flight-oriented state characteristic of patients" (King, 1999a, p.114) suffering from such extreme anxiety states as panic disorder or post-traumatic stress disorder (PTSD, King, 1999a).

8.0 Suggestions for Future Research

As has been discussed throughout this thesis, the present study has highlighted the need for future research into the long-term effects of caffeine consumption during adolescence. Today's adolescents consume caffeine in large quantities. However, there is little scientific understanding of what long-term effects this consumption may be having on their behaviour and mood (Reissig, et al., 2009). To gain a complete understanding of how caffeine may interfere with brain development, there are several areas of the study which could be adapted, modified or expanded.

Firstly, exposing subjects to a wider range of caffeine doses may provide a clearer picture of the long-term anxiogenic effects of caffeine. This may shed some light on some of this study's findings in the open field, in which the high dose group was not significantly different

from the control group in scores of ambulation and rearing. Incorporating more groups treated with a wider range of doses might identify where, among the varying doses, this shift in behaviour occurs.

This leads to another area which could guide future research. Some of the paradoxical results found in this study, for example in the open field test, indicated that the measures used for emotionality may not capture this construct fully, or take into account other reasons for a particular behaviour. This study's methods did not capture the possibility that impulsivity (for example, low emergence latencies in the emergence test) or escape behaviour (for example, rearing and ambulation in the open field test) may be driving some of the behaviours seen, particularly among the high dose group. Incorporating specific tests of impulsivity and escape behaviour in the behavioural test battery may help to identify some of the motivations behind these behaviours. It is possible to look at impulsivity as a construct through animal models. One study showed that impulsivity could be seen in animals that showed increased locomotor activity in the open field test, but displayed a greater change in behaviour following the introduction of a novel object (Evenden, 1999). Likewise, escape behaviours have been researched, and paradigms have been produced to measure these behaviours. An example of this was achieved by studying the behaviour of animals in a threatening environment after repetitive electrical stimulation of the superior colliculus (King, 1999b).

The period of caffeine administration in this study encompassed 11 days during late adolescence, from PND45-PND55. However, Smith (2003) states that in order to incorporate the entire adolescent period, dosing should occur between PND28-PND60. As human adolescents are consuming caffeine from early adolescence, if not earlier, it may be appropriate for future research to incorporate the earlier stages of this developmental period

as well. Many of the neurochemical changes which occur at a young age, take place during a critical window of time in the pre-pubertal stages (Andersen, 2003). This information would also suggest that encompassing the early stages of adolescence in the dosing period could potentially provide more accurate information on the long-term effects caffeine may have on brain development.

Middle and late adulthood were the age ranges tested in this study. Testing rats through all the stages of adulthood, for example from early adulthood through to late adulthood, or old age, may also increase our understanding of the drug's effects. As emotionality in rats may possibly increase with age (Bessa, et al., 2005; Imhof, et al., 1993), this could help to clarify some of the age-related effects seen in the results of this study.

While the open-field and emergence tests are both common behavioural paradigms used to test for emotionality, there are other appropriate tests which may provide more data on the behavioural consequences of caffeine exposure. For example, following completion of all the behavioural testing described in this thesis, the relative adrenal weights of a small sample of saline- and caffeine-treated rats were determined when they were about 10 months old. Higher adrenal weights have been associated with emotional reactivity (Henry & Stephens, 1980). It was found that males' adrenal weights were heavier after adolescent caffeine treatment (Anderson & Hughes, 2008). Further research into this finding should involve weighing the adrenal glands of a higher proportion of the subjects, and including subjects from all treatment groups.

Millan (2003) provides an explanation of experimental models of anxiety widely used in rodents, which is summarised below, in Figure 1. Clearly, the two tests of anxiety used in this

research (the open field and emergence tests) are potentially limited in the amount and types of emotionality (anxiety) that they may capture. The reliability of other anxiety measures is not commented on in this paper, but this would need to be considered if different tests of anxiety were to be employed.

I. "Trait", long-term anxious states
- (A) Rodent strains displaying high or low anxiety
- (B) Inter-individual differences within a defined strain
- (C) Chronic exposure to fear-provoking stimuli
- (D) Genetic models: transgenic and knock-out mice

II. "State", acute anxious states
- (A) Unconditioned
  - (1) Exploration (avoidance, conflict)
    - (i) Light–dark box
    - (ii) Hole board (nose pokes)
    - (iii) Elevated plus-maze (open arms vs. closed arms)
    - (iv) Open field (central squares vs. peripheral squares)
    - (v) Neophobia/emergence test (novel object)
  - (2) Interaction based
    - (i) Active social interaction (unfamiliar rat pairs)
    - (ii) Resident intruder
    - (iii) Ultrasonic vocalization (separation induced)
  - (3) Acute response to aversive stimuli (environment or brain stimulation)
    - (i) Freezing
    - (ii) Ultrasonic vocalization
    - (iii) Startle
    - (iv) Autonomic-cardiovascular parameters (arterial pressure, heart rate, endocrine secretion)
  - (4) Defensive behaviour to threatening stimuli
    - (i) Fear/defence battery
- (B) Conditioned
  - (1) Conflict procedures
    - (i) Geller–Seifter (operant, lever-pressing for reward)
    - (ii) Vogel Conflict Test
    - (iii) Conditioned suppression (no punishment during test session)
    - (iv) Safety-signal withdrawal (no punishment during test session)
    - (v) Conditioned place aversion
  - (2) Non-conflict procedures
    - (i) Fear-induced freezing, startle and ultrasonic vocalizations (re-exposure to aversive environment)
    - (ii) Shock-probe (burying of aversive object)
  - (3) Drug-discrimination
    - (i) Anxiogenic agents

Figure 1: Experimental models of anxiety widely used in rodents. Adapted from Millan (2003, p.87).

As adolescent caffeine consumption continues to increase, the number of adults who will have been exposed to caffeine during this age will also rise. If anxiety is a possible effect from exposure to caffeine in adolescence, this could have important clinical implications for the future. Anxiety is already extremely prevalent in today's society (Beidel & Stipelman, 2007), so broadening our knowledge base of how some of this anxiety may potentially be avoided earlier on in life would be beneficial, both clinically and socially. It seems clear through the animal literature, including this study, that while caffeine can be viewed as a weak teratogen (Nehlig & Debry, 1994), exposure to this drug at many stages in development does have lasting behavioural effects in rodents (Hughes & Beveridge, 1987, 1991; Hughes & Loader, 1996). Important to consider also, is that generalising animal studies to humans is not without its difficulties. One must consider the differing developmental pathways rats experience compared to humans from gestation and birth through to adulthood (Clancy, et al., 2007). Therefore, to completely understand the clinical implications of caffeine exposure during adolescence, studying the effects on humans directly should be undertaken. It does seem clear that clinical research on caffeine exposure during adolescence is needed.

9.0 Conclusions

Caffeine is one of the world's most widely used psychotropic drug (Hughes, 1996; Liguori, et al., 1997; Nehlig, et al., 1992; Weinberg & Bealer, 2001; Zahn & Rapoport, 1987), yet its methods of action and long-term effects are not understood fully. Children and adolescents are consuming caffeine regularly (Knight, et al., 2004; Reissig, et al., 2009; Skinner, et al., 2000). However their brains are undergoing considerable change during these vulnerable years (Andersen, 2003). There has been little research investigating the effects caffeine may

have on the developing adolescent brain. This study sought to investigate some of the long-term effects caffeine may have, when administered during the adolescent period.

The caffeine exposure may have led to functional changes in the brain, in particular in regions associated with adenosine and dopamine activity. A limitation of the present study was that analyses of neurochemical changes in the brain was not performed. Therefore there is no conclusive evidence of exactly how caffeine had exerted its effects in the brain.

What this research shows, despite its limitations, is that further research into adolescent caffeine use and its effects is warranted, and its clinical implications studied. This study found that rats exposed to caffeine during adolescence demonstrated some increases in emotionality. It is possible then, that people exposed to caffeine as adolescents, may show increased anxiety later in life.

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Appendices

Appendix 1

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UNIVERSITY OF CANTERBURY
Te Whare Wānanga o Waitaha
CHRISTCHURCH NEW ZEALAND

AEC Ref: 2006/14R

5 September 2006

Ms Nika Anderson
Psychology
UNIVERSITY OF CANTERBURY

Dear Ms Anderson

I am pleased to inform you that the Animal Ethics Committee has approved your application entitled: "Enduring behavioural effects in rats treated with caffeine during adolescence"

Approval has been granted:

(a) for the use of 30 male and 30 female rats
(b) for your research project to be undertaken over a period of one year from 4 September 2006 to 3 September 2007.

Please find enclosed a copy of the Animal Welfare (Records and Statistics) Regulations 1999 for your information.

Also enclosed is a copy of the MAF Animal Manipulation Statistical form (and list of Animal Type Codes and brief guideline notes) which you are required to complete and return to the Secretary of the AEC (Mrs Deborah Wekking, Level 6 Registry) 30 days after the completion of this project. If your project is ongoing and will not be finished during 2006 please note these trials are only reported at their completion, or at the end of each subsequent three year period. If no animals have been manipulated in your project please provide a "Nil" return.

Yours sincerely

Associate Professor Lou Reinisch
Chair, Animal Ethics Committee
Dean of Science

c.c. Animal Ethics Committee

Appendix 2

Raw Data

Open Field Raw Data
Testing period 1. Day 1.

	Cage No.	*Rat*							
Date	**MALES**		*Defaecation*	*Urination*	*Rearing (corners)*	*Rearing (outer)*	*Rearing (inner)*	*Freezing*	*Locomotion score*
04.10.06	High 1	Red #1	6	1	9	8	1	3	28
04.10.06		Blue #2	6	2	15	21	4	6	56
04.10.06		Green #3	6	0	19	14	7	3	59
04.10.06	High 2	Red #4	0	0	22	16	4	4	99
04.10.06		Blue #5	1	0	20	15	7	4	86
04.10.06		Green #6	3	0	14	12	5	7	80
04.10.06	High 3	Red #7	0	0	22	18	8	2	63
04.10.06		Blue #8	1	0	18	15	7	2	60
04.10.06		Green #9	2	0	24	19	7	3	77
02.10.06	Control 4	Red #1	0	2	24	13	4	1	57
02.10.06		Blue #2	2	0	15	5	2	1	65
02.10.06		Green #3	0	1	6	8	2	2	41
02.10.06	Control 5	Red #4	1	0	33	14	3	0	70
02.10.06		Blue #5	1	0	11	15	7	1	93
02.10.06		Green #6	4	0	15	7	10	0	66
02.10.06	Control 6	Red #7	2	1	14	13	2	1	63
02.10.06		Blue #8	0	2	18	10	3	0	75
02.10.06		Green#9	2	1	21	17	4	0	97
03.10.06	Low 7	Red #1	0	1	21	15	3	0	84
03.10.06		Blue #2	0	0	14	17	0	0	71
03.10.06		Green #3	0	1	19	10	1	0	92
03.10.06	Low 8	Red #4	0	1	21	15	0	1	54
03.10.06		Blue #5	2	1	12	13	7	2	77
03.10.06		Green #6	7	0	17	13	2	2	61
03.10.06	Low 9	Red #7	0	0	13	14	1	1	48
03.10.06		Blue #8	0	1	23	9	1	0	52
03.10.06		Green #9	0	2	17	20	4	0	91

Open Field Raw Data
Testing period 1. Day 1.

Date	**FEMALES**		*Defaecation*	*Urination*	*Rearing (corners)*	*Rearing (outer)*	*Rearing (inner)*	*Freezing*	*Locomotion score*
04.10.06	High 10	Red #10	0	0	15	15	0	3	70
04.10.06		Blue #11	0	0	33	17	8	1	94
04.10.06		Green #12	7	0	23	9	2	4	91
04.10.06	High 11	Red #13	1	0	18	20	1	1	113
04.10.06		Blue #14	0	1	21	9	1	4	82
04.10.06		Green #15	0	0	23	16	2	3	97
04.10.06	High 12	Red #16	4	0	14	12	0	3	108
04.10.06		Blue #17	0	0	28	10	5	2	77
04.10.06		Green #18	0	0	26	16	3	2	108
02.10.06	Control 13	Red #10	0	0	22	15	1	3	86
02.10.06		Blue #11	0	0	17	13	5	0	106
02.10.06		Green #12	1	0	14	17	0	0	84
02.10.06	Control 14	Red #13	0	0	14	12	3	0	74
02.10.06		Blue #14	0	0	23	15	2	2	90
02.10.06		Green #15	0	0	24	19	1	0	121
02.10.06	Control 15	Red #16	0	2	18	14	1	0	87
02.10.06		Blue #17	0	0	20	19	0	0	93
02.10.06		Green #18	0	0	19	23	0	0	93
03.10.06	Low 16	Red #10	0	0	23	11	0	1	77
03.10.06		Blue #11	0	0	23	10	2	1	96
03.10.06		Green #12	1	0	27	14	4	1	108
03.10.06	Low 17	Red #13	0	0	15	6	0	3	39
03.10.06		Blue #14	3	0	27	16	2	4	93
03.10.06		Green #15	0	0	19	18	1	6	79
03.10.06	Low 18	Red #16	0	0	18	15	2	1	80
03.10.06		Blue #17	0	0	19	9	6	2	103
03.10.06		Green #18	0	0	16	15	0	2	68

Emergence Test Raw Data
Testing period 1. Day 1.

	Cage No,	***Rat***			
Date	**MALES**		*Time to emerge (s)*	*No. Head pokes*	*Defaecation (Start Box)*
04.10.06	High 1	Red #1	300.00	14	0
04.10.06		Blue #2	36.97	6	0
04.10.06		Green #3	14.69	1	0
04.10.06	High 2	Red #4	12.40	0	0
04.10.06		Blue #5	4.31	0	0
04.10.06		Green #6	5.63	1	0
04.10.06	High 3	Red #7	13.66	0	0
04.10.06		Blue #8	36.66	3	0
04.10.06		Green #9	21.34	3	2
02.10.06	Control 4	Red #1	10.90	1	0
02.10.06		Blue #2	300.00	9	0
02.10.06		Green #3	27.10	2	1
02.10.06	Control 5	Red #4	15.30	1	0
02.10.06		Blue #5	14.70	0	0
02.10.06		Green #6	22.00	1	0
02.10.06	Control 6	Red #7	56.90	5	0
02.10.06		Blue #8	13.30	1	0
02.10.06		Green#9	13.90	1	0
03.10.06	Low 7	Red #1	300.00	15	1
03.10.06		Blue #2	15.09	1	0
03.10.06		Green #3	33.07	5	0
03.10.06	Low 8	Red #4	15.41	1	0
03.10.06		Blue #5	82.56	5	0
03.10.06		Green #6	61.41	5	0
03.10.06	Low 9	Red #7	35.60	1	0
03.10.06		Blue #8	300.00	14	0
03.10.06		Green #9	34.09	2	0

Emergence Test Raw Data
Testing period 1. Day 1.

Date	**FEMALES**		*Time to Emerge (s)*	*No. Head Pokes*	*Defaecation (Start Box)*
04.10.06	High 10	Red #10	23.25	2	0
04.10.06		Blue #11	22.34	3	0
04.10.06		Green #12	43.81	3	0
04.10.06	High 11	Red #13	52.12	5	0
04.10.06		Blue #14	33.19	3	0
04.10.06		Green #15	39.84	3	0
04.10.06	High 12	Red #16	8.18	2	0
04.10.06		Blue #17	21.25	1	0
04.10.06		Green #18	107.81	5	0
02.10.06	Control 13	Red #10	12.50	0	0
02.10.06		Blue #11	15.30	1	0
02.10.06		Green #12	21.20	1	0
02.10.06	Control 14	Red #13	53.60	3	0
02.10.06		Blue #14	76.80	6	0
02.10.06		Green #15	15.80	0	0
02.10.06	Control 15	Red #16	300.00	8	0
02.10.06		Blue #17	123.50	5	1
02.10.06		Green #18	31.60	2	0
03.10.06	Low 16	Red #10	182.84	15	0
03.10.06		Blue #11	75.84	5	0
03.10.06		Green #12	13.97	1	1
03.10.06	Low 17	Red #13	45.25	4	0
03.10.06		Blue #14	55.66	6	2
03.10.06		Green #15	26.44	2	0
03.10.06	Low 18	Red #16	22.78	2	0
03.10.06		Blue #17	90.53	5	0
03.10.06		Green #18	31.53	2	0

Open Field Raw Data
Testing period 1. Day 2.

	Cage No.	***Rat***							
Date	**MALES**		*Defaecation*	*Urination*	*Rearing (corners)*	*Rearing (outer)*	*Rearing (inner)*	*Freezing*	*Locomotion score*
08.10.06	High 1	Red #1	0	0	7	1	0	1	19
08.10.06		Blue #2	4	1	6	7	1	4	35
08.10.06		Green #3	8	3	9	4	1	2	42
08.10.06	High 2	Red #4	0	0	25	19	0	5	110
08.10.06		Blue #5	7	1	16	14	0	3	69
08.10.06		Green #6	7	1	13	9	0	5	73
08.10.06	High 3	Red #7	6	0	31	11	0	2	92
08.10.06		Blue #8	0	0	11	7	0	3	41
08.10.06		Green #9	0	1	17	9	0	1	57
06.10.06	Control 4	Red #1	3	1	23	18	0	0	70
06.10.06		Blue #2	0	1	12	11	0	3	59
06.10.06		Green #3	0	1	22	12	4	2	77
06.10.06	Control 5	Red #4	0	0	24	19	1	2	86
06.10.06		Blue #5	1	0	14	7	0	3	57
06.10.06		Green #6	0	0	15	12	0	4	45
06.10.06	Control 6	Red #7	1	0	6	6	0	2	27
06.10.06		Blue #8	0	0	20	9	0	2	78
06.10.06		Green#9	0	0	18	11	0	0	80
07.10.06	Low 7	Red #1	0	1	7	0	0	0	11
07.10.06		Blue #2	0	0	14	6	0	1	47
07.10.06		Green #3	0	0	11	9	0	2	39
07.10.06	Low 8	Red #4	0	0	13	7	0	2	47
07.10.06		Blue #5	6	1	11	9	1	1	54
07.10.06		Green #6	8	0	24	22	3	2	71
07.10.06	Low 9	Red #7	0	0	8	18	0	2	50
07.10.06		Blue #8	0	0	2	6	0	0	10
07.10.06		Green #9	0	0	13	4	0	0	35

Date	**FEMALES**		*Defaecation*	*Urination*	*Rearing (corners)*	*Rearing (outer)*	*Rearing (inner)*	*Freezing*	*Locomotion score*
08.10.06	High 10	Red #10	2	0	17	11	3	4	91
08.10.06		Blue #11	2	0	14	16	3	0	95
08.10.06		Green #12	5	1	20	18	1	1	101
08.10.06	High 11	Red #13	0	0	16	10	2	0	108
08.10.06		Blue #14	2	2	24	9	0	0	115
08.10.06		Green #15	1	1	15	3	0	1	49
08.10.06	High 12	Red #16	4	2	10	9	7	3	98
08.10.06		Blue #17	1	0	13	9	4	1	77
08.10.06		Green #18	0	0	12	15	1	2	104
06.10.06	Control 13	Red #10	0	0	18	12	0	2	40
06.10.06		Blue #11	0	0	15	13	2	2	81
06.10.06		Green #12	0	0	22	8	0	0	69
06.10.06	Control 14	Red #13	0	0	14	17	1	0	82
06.10.06		Blue #14	0	0	12	10	2	2	88
06.10.06		Green #15	0	1	1	1	0	0	29
06.10.06	Control 15	Red #16	1	0	17	3	0	0	62
06.10.06		Blue #17	0	0	17	14	0	0	78
06.10.06		Green #18	0	1	12	16	0	3	73
07.10.06	Low 16	Red #10	0	0	15	7	0	0	81
07.10.06		Blue #11	0	0	2	0	0	2	39
07.10.06		Green #12	0	0	4	0	0	1	14
07.10.06	Low 17	Red #13	0	0	7	3	0	1	35
07.10.06		Blue #14	5	1	25	13	5	0	109
07.10.06		Green #15	0	0	16	10	1	1	84
07.10.06	Low 18	Red #16	0	0	5	0	0	1	29
07.10.06		Blue #17	0	0	20	18	4	3	108
07.10.06		Green #18	0	0	6	6	0	1	43

Emergence Test Raw Data
Testing period 1. Day 2.

	Cage No.	***Rat***			
Date	**MALES**		*Time to emerge (s)*	*No. Head pokes*	*Defaecation (Start Box)*
08.10.06	High 1	Red #1	300.00	6	0
08.10.06		Blue #2	12.63	2	0
08.10.06		Green #3	171.03	13	0
08.10.06	High 2	Red #4	6.91	1	0
08.10.06		Blue #5	17.22	2	0
08.10.06		Green #6	12.19	1	0
08.10.06	High 3	Red #7	22.88	3	0
08.10.06		Blue #8	30.09	4	0
08.10.06		Green #9	10.22	1	0
06.10.06	Control 4	Red #1	37.68	6	0
06.10.06		Blue #2	300.00	7	2
06.10.06		Green #3	5.78	1	0
06.10.06	Control 5	Red #4	13.09	1	0
06.10.06		Blue #5	300.00	15	0
06.10.06		Green #6	58.22	2	0
06.10.06	Control 6	Red #7	216.37	6	0
06.10.06		Blue #8	10.00	1	0
06.10.06		Green#9	6.31	1	0
07.10.06	Low 7	Red #1	300.00	6	0
07.10.06		Blue #2	151.62	8	0
07.10.06		Green #3	86.78	6	0
07.10.06	Low 8	Red #4	69.37	5	0
07.10.06		Blue #5	65.50	6	0
07.10.06		Green #6	300.00	17	0
07.10.06	Low 9	Red #7	300.00	10	0
07.10.06		Blue #8	300.00	7	0
07.10.06		Green #9	160.06	10	0

Emergence Test Raw Data
Testing Period 1. Day 2.

Date	**FEMALES**		*Time to Emerge (s)*	*No. Head Pokes*	*Defaecation (Start Box)*
08.10.06	High 10	Red #10	86.59	5	0
08.10.06		Blue #11	237.72	9	0
08.10.06		Green #12	16.75	3	0
08.10.06	High 11	Red #13	82.79	3	0
08.10.06		Blue #14	14.85	2	0
08.10.06		Green #15	30.94	2	0
08.10.06	High 12	Red #16	20.06	2	0
08.10.06		Blue #17	24.81	3	0
08.10.06		Green #18	15.28	1	0
06.10.06	Control 13	Red #10	16.78	2	0
06.10.06		Blue #11	18.78	1	0
06.10.06		Green #12	57.35	6	0
06.10.06	Control 14	Red #13	93.85	2	0
06.10.06		Blue #14	55.44	1	0
06.10.06		Green #15	120.19	9	0
06.10.06	Control 15	Red #16	113.66	4	1
06.10.06		Blue #17	71.47	6	0
06.10.06		Green #18	42.18	4	0
07.10.06	Low 16	Red #10	300.00	11	0
07.10.06		Blue #11	10.62	1	0
07.10.06		Green #12	300.00	14	1
07.10.06	Low 17	Red #13	191.81	4	0
07.10.06		Blue #14	112.18	10	0
07.10.06		Green #15	79.97	8	0
07.10.06	Low 18	Red #16	300.00	13	0
07.10.06		Blue #17	97.09	7	0
07.10.06		Green #18	300.00	10	0

Open Field Raw Data.
Testing period 1. Day 3.

	Cage No.	***Rat***							
Date	**MALES**		*Defaecation*	*Urination*	*Rearing (corners)*	*Rearing (outer)*	*Rearing (inner)*	*Freezing*	*Locomotion score*
12.10.06	High 1	Red #1	0	1	9	6	0	2	27
12.10.06		Blue #2	5	2	17	17	0	5	66
12.10.06		Green #3	2	1	19	10	2	4	55
12.10.06	High 2	Red #4	0	0	14	9	0	3	66
12.10.06		Blue #5	0	0	20	10	3	6	80
12.10.06		Green #6	8	0	8	16	0	6	67
12.10.06	High 3	Red #7	0	1	30	14	1	2	75
12.10.06		Blue #8	0	0	12	15	0	0	48
12.10.06		Green #9	0	0	15	15	0	0	41
10.10.06	Control 4	Red #1	4	1	20	29	1	1	88
10.10.06		Green #3	0	0	16	12	0	2	55
10.10.06	Control 5	Red #4	0	0	24	18	1	1	79
10.10.06		Blue #5	0	0	17	26	0	3	78
10.10.06		Green #6	0	0	14	11	1	1	46
10.10.06	Control 6	Red #7	0	0	5	1	0	3	16
10.10.06		Blue #8	0	0	17	18	0	2	74
10.10.06		Green#9	6	0	19	8	1	1	71
11.10.06	Low 7	Red #1	0	0	2	1	0	1	16
11.10.06		Blue #2	0	0	6	5	1	3	42
11.10.06		Green #3	0	0	21	8	1	2	66
11.10.06	Low 8	Red #4	0	0	12	4	0	2	42
11.10.06		Blue #5	3	1	8	4	0	2	36
11.10.06		Green #6	4	0	17	23	2	1	73
11.10.06	Low 9	Red #7	5	1	11	12	0	2	47
11.10.06		Blue #8	8	1	17	2	3	1	36
11.10.06		Green #9	0	2	12	5	0	3	41

Open Field Raw Data.
Testing period 1. Day 3.

Date	**FEMALES**		*Defaecation*	*Urination*	*Rearing (corners)*	*Rearing (outer)*	*Rearing (inner)*	*Freezing*	*Locomotion score*
12.10.06	High 10	Red #10	0	0	20	18	2	1	132
12.10.06		Blue #11	0	0	18	9	1	6	90
12.10.06		Green #12	7	1	14	18	3	3	90
12.10.06	High 11	Red #13	0	0	14	18	3	3	125
12.10.06		Blue #14	0	1	23	11	0	4	113
12.10.06		Green #15	0	0	22	10	0	0	77
12.10.06	High 12	Red #16	4	1	13	15	3	4	92
12.10.06		Blue #17	2	0	17	12	4	0	81
12.10.06		Green #18	0	1	23	14	6	1	110
10.10.06	Control13	Red #10	0	0	21	26	3	0	119
10.10.06		Blue #11	0	0	22	28	3	0	99
10.10.06		Green #12	0	1	20	17	2	2	113
10.10.06	Control 14	Red #13	0	0	10	7	0	5	59
10.10.06		Blue #14	0	0	18	16	4	2	95
10.10.06		Green #15	0	0	15	8	0	0	68
10.10.06	Control 15	Red #16	0	0	11	9	0	1	60
10.10.06		Blue #17	0	0	24	16	0	1	110
10.10.06		Green #18	0	0	23	9	0	0	56
11.10.06	Low 16	Red #10	0	1	18	17	0	4	117
11.10.06		Blue #11	0	0	16	7	0	2	90
11.10.06		Green #12	2	0	12	1	1	2	34
11.10.06	Low 17	Red #13	0	0	19	10	0	3	72
11.10.06		Blue #14	6	1	29	25	3	0	117
11.10.06		Green #15	0	0	17	15	2	4	97
11.10.06	L18	Red #16	0	0	13	15	0	0	75
11.10.06		Blue #17	3	2	18	20	2	4	109
11.10.06		Green #18	0	0	18	18	5	2	95

Emergence Test Raw Data.
Testing period 1. Day 3.

	Cage No.	***Rat***			
Date	**MALES**		*Time to emerge (s)*	*No. Head pokes*	*Defaecation (Start Box)*
12.10.06	High 1	Red #1	300.00	1	0
12.10.06		Blue #2	36.28	3	0
12.10.06		Green #3	300.00	11	0
12.10.06	High 2	Red #4	7.62	1	0
12.10.06		Blue #5	76.81	4	0
12.10.06		Green #6	62.88	5	0
12.10.06	High 3	Red #7	80.56	5	0
12.10.06		Blue #8	59.85	4	0
12.10.06		Green #9	13.78	2	0
10.10.06	Control 4	Red #1	14.38	1	0
10.10.06		Blue #2	300.00	6	0
10.10.06		Green #3	117.44	12	0
10.10.06	Control 5	Red #4	6.00	1	0
10.10.06		Blue #5	300.00	4	0
10.10.06		Green #6	300.00	11	0
10.10.06	Control 6	Red #7	300.00	5	0
10.10.06		Blue #8	6.35	1	0
10.10.06		Green#9	4.25	1	0
11.10.06	Low 7	Red #1	300.00	7	0
11.10.06		Blue #2	47.90	2	0
11.10.06		Green #3	158.53	12	0
11.10.06	Low 8	Red #4	69.16	2	0
11.10.06		Blue #5	100.41	5	0
11.10.06		Green #6	131.69	8	0
11.10.06	Low 9	Red #7	247.03	6	0
11.10.06		Blue #8	300.00	7	0
11.10.06		Green #9	55.97	6	0

Emergence Test Raw Data.
Testing period 1. Day 3.

Date	**FEMALES**		*Time to Emerge (s)*	*No. Head Pokes*	*Defaecation (Start Box)*
12.10.06	High 10	Red #10	9.40	1	0
12.10.06		Blue #11	39.94	1	0
12.10.06		Green #12	89.62	4	0
12.10.06	High 11	Red #13	17.82	1	0
12.10.06		Blue #14	5.72	1	0
12.10.06		Green #15	72.82	5	0
12.10.06	High 12	Red #16	47.97	3	0
12.10.06		Blue #17	19.35	1	0
12.10.06		Green #18	17.35	1	0
10.10.06	Control 13	Red #10	13.06	2	0
10.10.06		Blue #11	7.97	1	0
10.10.06		Green #12	9.78	1	0
10.10.06	Control 14	Red #13	97.50	7	0
10.10.06		Blue #14	12.00	1	0
10.10.06		Green #15	300.00	9	0
10.10.06	Control 15	Red #16	242.04	7	0
10.10.06		Blue #17	8.59	1	0
10.10.06		Green #18	85.94	5	0
11.10.06	Low 16	Red #10	300.00	13	3
11.10.06		Blue #11	26.12	2	0
11.10.06		Green #12	197.44	1	0
11.10.06	Low 17	Red #13	93.22	4	0
11.10.06		Blue #14	10.81	1	0
11.10.06		Green #15	47.75	6	0
11.10.06	Low 18	Red #16	61.88	5	0
11.10.06		Blue #17	8.85	1	0
11.10.06		Green #18	179.47	9	0

Open Field Raw Data.
Testing period 2. Day 1.

	Cage No.	***Rat***							
Date	**MALES**		*Defaecation*	*Urination*	*Rearing (corners)*	*Rearing (outer)*	*Rearing (inner)*	*Freezing*	*Locomotion score*
23.11.06	High 1	Red #1	0	1	9	15	2	3	13
23.11.06		Blue #2	0	2	19	24	0	0	65
23.11.06		Green #3	3	1	8	14	7	2	46
23.11.06	High 2	Red #4	0	0	28	8	1	2	59
23.11.06		Blue #5	3	0	16	14	2	0	85
23.11.06		Green #6	7	2	15	14	2	1	59
23.11.06	High 3	Red #7	3	2	17	20	3	1	58
23.11.06		Blue #8	0	0	17	12	0	2	39
23.11.06		Green #9	0	0	16	1	0	1	29
17.11.06	Control 4	Red #1	0	1	7	13	0	2	46
17.11.06		Blue #2	0	0	8	4	0	3	30
17.11.06	Control 5	Red #3	0	1	19	20	4	0	61
17.11.06		Blue #4	6	2	15	21	0	1	68
17.11.06		Green #5	0	0	9	11	6	1	45
17.11.06	Control 6	Red #6	0	2	7	0	0	0	10
17.11.06		Blue #7	0	0	14	10	3	0	49
17.11.06		Green#9	2	0	14	12	1	2	71
22.11.06	Low 7	Red #1	0	4	5	2	0	1	23
22.11.06		Blue #2	0	0	16	9	0	0	56
22.11.06		Green #3	0	0	12	3	0	1	25
22.11.06	Low 8	Red #4	0	0	19	4	0	1	63
22.11.06		Blue #5	4	1	9	15	0	0	49
22.11.06		Green #6	6	0	18	22	2	1	55
22.11.06	Low 9	Red #7	0	0	19	19	4	0	72
22.11.06		Blue #8	2	2	20	8	0	0	46
22.11.06		Green #9	0	0	29	17	0	0	60

Open Field Raw Data.
Testing period 2. Day 1.

Date	**FEMALES**		*Defaecation*	*Urination*	*Rearing (corners)*	*Rearing (outer)*	*Rearing (inner)*	*Freezing*	*Locomotion score*
23.11.06	High 10	Red #10	4	0	21	26	1	1	81
23.11.06		Blue #11	5	0	17	18	3	1	74
23.11.06		Green #12	4	2	13	19	3	0	84
23.11.06	High 11	Red #13	0	1	21	20	4	2	121
23.11.06		Blue #14	0	0	28	14	4	2	123
23.11.06		Green #15	2	0	26	15	1	0	79
23.11.06	High 12	Red #16	3	2	14	20	4	3	99
23.11.06		Blue #17	5	1	23	12	1	0	67
23.11.06		Green #18	0	0	28	26	2	1	102
17.11.06	Control 13	Red #10	0	0	23	39	3	0	103
17.11.06		Blue #11	0	2	22	21	0	0	85
17.11.06		Green #12	2	0	22	30	9	0	103
17.11.06	Control 14	Red #13	0	0	26	29	2	0	88
17.11.06		Blue #14	0	0	15	14	1	0	73
17.11.06		Green #15	0	0	7	7	1	1	49
17.11.06	Control 15	Red #16	5	1	17	26	7	0	85
17.11.06		Blue #17	0	0	12	15	5	2	87
17.11.06		Green #18	0	0	22	22	5	0	79
22.11.06	Low 16	Red #10	0	0	25	30	0	1	97
22.11.06		Blue #11	3	1	21	19	1	2	91
22.11.06		Green #12	1	0	12	7	3	1	28
22.11.06	Low 17	Red #13	0	0	16	3	0	2	45
22.11.06		Blue #14	0	0	14	5	1	1	93
22.11.06		Green #15	0	1	17	12	2	0	77
22.11.06	Low 18	Red #16	0	3	5	5	1	1	55
22.11.06		Blue #17	4	0	8	8	3	0	66
22.11.06		Green #18	0	0	16	7	0	0	50

Emergence Test Raw Data.
Testing period 2. Day 1.

	Cage No.	***Rat***			
Date	**MALES**		*Time to emerge (s)*	*No. Head pokes*	*Defaecation (Start Box)*
23.11.06	High 1	Red #1	300.00	4	5
23.11.06		Blue #2	17.00	2	0
23.11.06		Green #3	300.00	8	2
23.11.06	High 2	Red #4	43.15	3	0
23.11.06		Blue #5	35.63	5	0
23.11.06		Green #6	40.47	5	0
23.11.06	High 3	Red #7	50.97	5	0
23.11.06		Blue #8	14.28	2	0
23.11.06		Green #9	122.53	10	0
17.11.06	Control 4	Red #1	160.60	5	0
17.11.06		Blue #2	300.00	4	0
17.11.06	Control 5	Red #3	110.50	5	0
17.11.06		Blue #4	300.00	9	0
17.11.06		Green #5	300.00	6	0
17.11.06	Control 6	Red #6	300.00	6	0
17.11.06		Blue #7	17.93	1	0
17.11.06		Green#9	80.78	6	0
22.11.06	Low 7	Red #1	300.00	0	0
22.11.06		Blue #2	300.00	6	0
22.11.06		Green #3	300.00	9	0
22.11.06	Low 8	Red #4	90.34	4	0
22.11.06		Blue #5	300.00	8	2
22.11.06		Green #6	130.53	8	2
22.11.06	Low 9	Red #7	300.00	6	0
22.11.06		Blue #8	300.00	1	0
22.11.06		Green #9	300.00	9	0

Emergence Test Raw Data.
Testing period 2. Day 1.

Date	**FEMALES**		*Time to Emerge (s)*	*No. Head Pokes*	*Defaecation (Start Box)*
23.11.06	High 10	Red #10	95.16	9	0
23.11.06		Blue #11	40.22	4	0
23.11.06		Green #12	30.25	3	0
23.11.06	High 11	Red #13	179.56	4	0
23.11.06		Blue #14	4.25	0	0
23.11.06		Green #15	45.53	1	0
23.11.06	High 12	Red #16	169.81	9	0
23.11.06		Blue #17	22.28	2	0
23.11.06		Green #18	8.81	1	0
17.11.06	Control 13	Red #10	13.69	3	0
17.11.06		Blue #11	57.66	2	0
17.11.06		Green #12	126.84	6	0
17.11.06	Control 14	Red #13	300.00	6	0
17.11.06		Blue #14	151.13	4	0
17.11.06		Green #15	248.82	7	1
17.11.06	Control 15	Red #16	300.00	5	0
17.11.06		Blue #17	300.00	12	0
17.11.06		Green #18	300.00	16	0
22.11.06	Low 16	Red #10	300.00	11	2
22.11.06		Blue #11	20.97	3	0
22.11.06		Green #12	300.00	5	3
22.11.06	Low 17	Red #13	300.00	11	0
22.11.06		Blue #14	36.43	4	0
22.11.06		Green #15	62.93	7	0
22.11.06	Low 18	Red #16	300.00	7	0
22.11.06		Blue #17	300.00	14	0
22.11.06		Green #18	300.00	13	0

Open Field Raw Data.
Testing period 2. Day 2.

	Cage No.	***Rat***							
Date	**MALES**		*Defaecation*	*Urination*	*Rearing (corners)*	*Rearing (outer)*	*Rearing (inner)*	*Freezing*	*Locomotion score*
28.11.06	High 1	Red #1	0	0	7	3	1	0	18
28.11.06		Blue #2	0	0	23	19	4	2	86
28.11.06		Green #3	0	0	11	20	11	0	59
28.11.06	High 2	Red #4	0	0	21	14	0	0	73
28.11.06		Blue #5	0	1	20	5	1	1	73
28.11.06		Green #6	6	2	16	18	2	3	71
28.11.06	High 3	Red #7	3	0	29	24	7	1	81
28.11.06		Blue #8	0	0	10	11	2	1	54
28.11.06		Green #9	0	0	17	14	0	0	36
25.11.06	Control 4	Red #1	2	1	17	13	0	0	38
25.11.06		Blue #2	0	1	8	3	1	1	37
25.11.06	Control 5	Red #3	0	0	18	12	0	3	57
25.11.06		Blue #4	0	3	21	24	7	0	98
25.11.06		Green #5	0	0	11	17	0	1	44
25.11.06	Control 6	Red #6	4	0	7	1	0	1	14
25.11.06		Blue #7	0	0	19	15	0	0	56
25.11.06		Green#8	0	0	17	11	0	2	80
26.11.06	Low 7	Red #1	0	3	5	0	0	1	26
26.11.06		Blue #2	0	0	18	14	0	1	70
26.11.06		Green #3	0	0	10	8	0	2	47
26.11.06	Low 8	Red #4	0	0	15	9	3	3	60
26.11.06		Blue #5	3	0	9	9	0	2	43
26.11.06		Green #6	4	1	11	25	0	4	48
26.11.06	Low 9	Red #7	0	0	16	16	1	1	49
26.11.06		Blue #8	0	0	14	5	1	0	22
26.11.06		Green #9	0	0	22	12	3	1	57

Open Field Raw Data.
Testing period 2. Day 2.

Date	**FEMALES**		*Defaecation*	*Urination*	*Rearing (corners)*	*Rearing (outer)*	*Rearing (inner)*	*Freezing*	*Locomotion score*
28.11.06	High 10	Red #10	0	0	18	21	5	1	118
28.11.06		Blue #11	0	0	21	23	0	2	98
28.11.06		Green #12	5	2	15	12	0	1	73
28.11.06	High 11	Red #13	0	0	23	18	5	2	100
28.11.06		Blue #14	0	1	29	18	1	1	125
28.11.06		Green #15	0	0	20	24	6	0	96
28.11.06	High 12	Red #16	7	3	11	6	1	0	60
28.11.06		Blue #17	0	0	17	8	1	0	60
28.11.06		Green #18	0	0	22	20	3	0	104
25.11.06	Control 13	Red #10	0	0	24	23	3	0	99
25.11.06		Blue #11	0	0	24	17	1	0	91
25.11.06		Green #12	0	2	16	23	2	1	92
25.11.06	Control 14	Red #13	0	0	15	18	2	1	59
25.11.06		Blue #14	0	0	16	17	4	1	87
25.11.06		Green #15	0	0	14	8	1	0	67
25.11.06	Control 15	Red #16	0	0	17	19	0	1	87
25.11.06		Blue #17	0	0	16	16	0	1	107
25.11.06		Green #18	0	0	17	14	1	1	76
26.11.06	Low 16	Red #10	0	0	17	23	2	4	81
26.11.06		Blue #11	0	0	13	8	2	3	75
26.11.06		Green #12	0	0	4	1	1	2	29
26.11.06	Low 17	Red #13	0	0	16	11	3	0	64
26.11.06		Blue #14	0	0	13	10	2	5	84
26.11.06		Green #15	0	0	28	27	2	1	87
26.11.06	Low 18	Red #16	0	0	12	13	2	2	65
26.11.06		Blue #17	6	0	12	13	2	1	73
26.11.06		Green #18	0	0	11	9	1	0	70

Emergence Test Raw Data.
Testing period 2. Day 2.

	Cage No.	***Rat***			
Date	**MALES**		*Time to emerge (s)*	*No. Head pokes*	*Defaecation (Start Box)*
28.11.06	High 1	Red #1	300.00	4	0
28.11.06		Blue #2	9.44	2	0
28.11.06		Green #3	300.00	9	1
28.11.06	High 2	Red #4	61.72	3	0
28.11.06		Blue #5	6.53	0	0
28.11.06		Green #6	24.19	1	0
28.11.06	High 3	Red #7	28.84	1	0
28.11.06		Blue #8	11.06	0	0
28.11.06		Green #9	26.94	2	0
25.11.06	Control 4	Red #1	35.63	3	0
25.11.06		Blue #2	300.00	5	0
25.11.06	Control 5	Red #3	29.78	3	0
25.11.06		Blue #4	60.60	6	0
25.11.06		Green #5	189.21	17	0
25.11.06	Control 6	Red #6	118.19	7	0
25.11.06		Blue #7	83.59	6	0
25.11.06		Green#8	70.22	5	0
26.11.06	Low 7	Red #1	300.00	1	0
26.11.06		Blue #2	15.84	1	0
26.11.06		Green #3	64.50	5	0
26.11.06	Low 8	Red #4	27.44	2	0
26.11.06		Blue #5	300.00	15	0
26.11.06		Green #6	52.59	5	0
26.11.06	Low 9	Red #7	26.13	2	0
26.11.06		Blue #8	40.10	4	0
26.11.06		Green #9	300.00	19	0

Emergence Test Raw Data.
Testing period 2. Day 2.

Date	**FEMALES**		*Time to Emerge (s)*	*No. Head Pokes*	*Defaecation (Start Box)*
28.11.06	High 10	Red #10	35.34	5	0
28.11.06		Blue #11	12.59	1	0
28.11.06		Green #12	52.19	1	0
28.11.06	High 11	Red #13	102.31	5	0
28.11.06		Blue #14	77.03	5	0
28.11.06		Green #15	300.00	13	0
28.11.06	High 12	Red #16	77.19	3	0
28.11.06		Blue #17	142.72	9	0
28.11.06		Green #18	6.41	1	0
25.11.06	Control 13	Red #10	5.69	0	0
25.11.06		Blue #11	26.38	1	0
25.11.06		Green #12	37.75	5	0
25.11.06	Control 14	Red #13	57.72	2	0
25.11.06		Blue #14	129.78	7	0
25.11.06		Green #15	83.03	5	0
25.11.06	Control 15	Red #16	248.25	4	0
25.11.06		Blue #17	16.03	2	0
25.11.06		Green #18	300.00	9	0
26.11.06	Low 16	Red #10	300.00	17	0
26.11.06		Blue #11	12.59	0	0
26.11.06		Green #12	29.69	2	0
26.11.06	Low 17	Red #13	300.00	16	0
26.11.06		Blue #14	58.47	4	0
26.11.06		Green #15	38.47	5	0
26.11.06	Low 18	Red #16	205.47	13	0
26.11.06		Blue #17	165.66	7	0
26.11.06		Green #18	21.53	2	0

Open Field Raw Data.
Testing period 2. Day 3.

	Cage No.	***Rat***							
Date	**MALES**		*Defaecation*	*Urination*	*Rearing (corners)*	*Rearing (outer)*	*Rearing (inner)*	*Freezing*	*Locomotion score*
01.12.06	High 1	Red #1	4	0	17	14	2	1	31
01.12.06		Blue #2	0	0	25	14	4	5	77
01.12.06		Green #3	1	0	20	22	1	2	61
01.12.06	High 2	Red #4	0	0	33	12	0	1	107
01.12.06		Blue #5	0	0	19	16	3	6	85
01.12.06		Green #6	1	1	6	4	0	3	43
01.12.06	High 3	Red #7	3	0	36	20	1	1	92
01.12.06		Blue #8	0	0	23	18	0	0	74
01.12.06		Green #9	0	0	19	12	0	0	49
29.11.06	Control 4	Red #1	0	0	12	18	1	0	42
29.11.06		Blue #2	0	2	9	4	0	1	44
29.11.06	Control 5	Blue #4	0	0	14	27	3	0	90
29.11.06		Green #5	0	0	15	22	2	4	55
29.11.06	Control 6	Red #6	0	0	23	12	2	1	57
29.11.06		Blue #7	0	0	24	9	1	2	64
29.11.06		Green#9	0	0	15	6	1	2	52
30.11.06	Low 7	Red #1	0	2	9	2	0	3	23
30.11.06		Blue #2	0	0	18	12	2	3	78
30.11.06		Green #3	0	1	10	20	4	3	74
30.11.06	Low 8	Red #4	0	0	18	9	3	1	66
30.11.06		Blue #5	2	1	14	15	2	1	66
30.11.06		Green #6	7	2	19	25	7	3	75
30.11.06	Low 9	Red #7	0	0	14	13	4	3	66
30.11.06		Blue #8	0	1	25	14	2	0	52
30.11.06		Green #9	0	0	18	14	2	1	67

Open Field Raw Data.
Testing period 2. Day 3. .

Date	**FEMALES**		*Defaecation*	*Urination*	*Rearing (corners)*	*Rearing (outer)*	*Rearing (inner)*	*Freezing*	*Locomotion score*
01.12.06	High 10	Red #10	0	0	21	31	6	3	115
01.12.06		Blue #11	0	0	27	27	8	4	135
01.12.06		Green #12	7	2	18	20	6	6	129
01.12.06	High 11	Red #13	0	0	18	11	2	1	102
01.12.06		Blue #14	0	2	24	18	1	0	128
01.12.06		Green #15	0	0	21	20	7	1	119
01.12.06	High 12	Red #16	6	2	14	13	10	5	95
01.12.06		Blue #17	0	0	17	18	0	0	78
01.12.06		Green #18	0	0	20	12	7	4	108
29.11.06	Control 13	Red #10	0	0	25	28	5	0	119
29.11.06		Blue #11	0	2	28	16	3	2	127
29.11.06		Green #12	0	0	26	21	5	0	134
29.11.06	Control 14	Red #13	0	0	17	26	8	1	90
29.11.06		Blue #14	0	0	24	25	3	0	109
29.11.06		Green #15	0	0	20	11	1	0	77
29.11.06	Control 15	Red #16	0	0	23	15	0	0	86
29.11.06		Blue #17	0	0	16	7	2	0	81
29.11.06		Green #18	0	0	14	15	1	0	99
30.11.06	Low 16	Red #10	0	0	22	26	2	2	107
30.11.06		Blue #11	0	0	17	15	0	1	101
30.11.06		Green #12	0	0	15	11	1	1	61
30.11.06	Low 17	Red #13	0	0	19	20	3	0	87
30.11.06		Blue #14	4	1	14	22	9	2	105
30.11.06		Green #15	0	1	18	22	2	0	109
30.11.06	Low 18	Red #16	0	0	15	17	1	1	73
30.11.06		Blue #17	1	1	10	22	8	2	108
30.11.06		Green #18	0	1	15	12	3	1	94

Emergence Test Raw Data.
Testing period 2. Day 3.

	Cage No.	***Rat***			
Date	**MALES**		*Time to emerge (s)*	*No. Head pokes*	*Defaecation (Start Box)*
01.12.06	High 1	Red #1	300.00	5	0
01.12.06		Blue #2	7.62	1	0
01.12.06		Green #3	72.16	5	4
01.12.06	High 2	Red #4	37.66	2	0
01.12.06		Blue #5	24.97	3	0
01.12.06		Green #6	56.03	4	0
01.12.06	High 3	Red #7	22.63	3	0
01.12.06		Blue #8	74.01	7	0
01.12.06		Green #9	26.59	2	0
29.11.06	Control 4	Red #1	61.87	1	0
29.11.06		Blue #2	55.87	5	0
29.11.06	Control 5	Blue #4	105.32	1	0
29.11.06		Green #5	45.16	7	0
29.11.06	Control 6	Red #6	76.97	3	0
29.11.06		Blue #7	21.82	1	0
29.11.06		Green#9	35.40	3	0
30.11.06	Low 7	Red #1	274.40	4	0
30.11.06		Blue #2	57.84	5	0
30.11.06		Green #3	21.65	3	0
30.11.06	Low 8	Red #4	12.34	1	0
30.11.06		Blue #5	32.00	6	0
30.11.06		Green #6	24.50	6	0
30.11.06	Low 9	Red #7	83.34	6	0
30.11.06		Blue #8	116.59	5	0
30.11.06		Green #9	300.00	13	0

Emergence Test Raw Data.
Testing period 2. Day 3.

Date	**FEMALES**		*Time to Emerge*	*No. Head Pokes*	*Defaecation (Start Box)*
01.12.06	High 10	Red #10	67.06	5	0
01.12.06		Blue #11	28.62	3	0
01.12.06		Green #12	37.22	3	0
01.12.06	High 11	Red #13	6.78	0	0
01.12.06		Blue #14	15.35	0	0
01.12.06		Green #15	37.56	6	0
01.12.06	High 12	Red #16	28.12	3	0
01.12.06		Blue #17	48.31	3	0
01.12.06		Green #18	8.59	2	0
29.11.06	Control 13	Red #10	6.63	0	0
29.11.06		Blue #11	5.47	1	0
29.11.06		Green #12	16.06	2	0
29.11.06	Control 14	Red #13	300.00	7	0
29.11.06		Blue #14	29.44	3	0
29.11.06		Green #15	66.94	9	0
29.11.06	Control 15	Red #16	34.75	3	0
29.11.06		Blue #17	47.63	5	0
29.11.06		Green #18	58.50	3	0
30.11.06	Low 16	Red #10	300.00	24	0
30.11.06		Blue #11	19.87	2	0
30.11.06		Green #12	300.00	11	0
30.11.06	Low 17	Red #13	36.09	3	0
30.11.06		Blue #14	15.60	2	0
30.11.06		Green #15	25.34	3	0
30.11.06	Low 18	Red #16	61.63	6	0
30.11.06		Blue #17	59.34	3	0
30.11.06		Green #18	23.50	2	0

Behavioural Sampling Raw Data of Open Field Centre and Corner Occupancy.

Rat #	*Testing1 Day1*		*Testing1 Day2*		*Testing1 Day3*		*Testing2 Day1*		*Testing2 Day2*		*Testing2 Day3*	
	Centre	Corner	Centre	Corner	Centre	Corner	Centre	Corner	Centre	Corner	Centre	Corner
H1R	8	33	0	84	0	69	3	75	0	88	1	63
H1B	12	48	2	68	4	51	1	38	3	45	5	52
H1G	13	46	3	71	3	46	9	27	21	25	3	47
H2R	11	46	0	60	0	54	2	75	2	59	2	71
H2B	12	50	0	65	3	44	6	60	1	57	6	49
H2G	13	41	1	58	6	33	5	43	1	47	11	60
H3R	7	52	2	54	2	62	2	42	6	57	4	69
H3B	4	44	1	62	0	47	1	52	3	48	1	56
H3G	9	47	1	64	1	46	1	77	1	72	2	57
H10R	8	47	0	58	4	51	3	47	7	39	8	32
H10B	11	53	7	60	5	73	7	56	3	40	7	39
H10G	12	48	1	48	6	39	7	36	3	48	17	37
H11R	5	46	6	55	6	32	12	50	12	45	13	42
H11B	7	53	3	63	2	61	9	53	2	47	0	49
H11G	9	62	0	80	0	62	1	65	5	47	7	43
H12R	7	44	8	61	4	53	15	37	6	44	15	37
H12B	6	59	5	63	8	55	3	67	2	69	5	53
H12G	4	63	3	50	9	58	3	58	3	52	5	65
C4R	8	54	0	50	5	28	8	58	3	60	0	50
C4B	14	51	4	52	1	87	4	55	3	81	1	60
C4G	5	58	6	48	0	63	-	-	-	-	-	-
C5R	7	57	3	43	4	60	11	47	2	50	-	-
C5B	13	46	1	66	1	35	4	45	11	40	14	28
C5G	24	37	0	58	4	56	10	52	1	25	3	35
C6R	9	39	0	58	0	94	2	90	0	41	4	69
C6B	7	49	1	59	5	31	6	44	7	53	3	60
C6G	10	58	5	60	3	55	5	53	5	57	3	71
C13R	5	52	1	55	10	37	4	41	6	38	6	43
C13B	9	43	9	39	6	47	7	48	5	52	6	48
C13G	10	40	1	64	3	48	14	39	3	48	3	49
C14R	4	52	8	56	3	68	4	48	4	49	9	37
C14B	3	52	6	65	7	49	3	43	6	39	9	46
C14G	5	42	2	29	5	55	3	39	2	37	8	48
C15R	8	53	0	61	2	67	9	36	2	53	0	48
C15B	3	46	0	67	1	51	19	38	4	42	3	52
C15G	8	45	17	39	1	59	12	44	1	48	3	49
L7R	7	49	0	95	0	55	0	79	1	90	1	84
L7B	8	42	0	63	7	53	2	58	0	60	4	57
L7G	8	47	0	45	1	66	0	77	3	62	3	41
L8R	8	52	0	61	3	78	3	63	6	55	4	66
L8B	18	33	3	45	1	74	6	32	0	32	5	43
L8G	10	40	3	42	7	35	5	39	7	26	11	36
L9R	15	45	0	26	2	47	7	47	6	54	8	38
L9B	1	69	0	53	3	80	3	61	1	81	4	60

	Centre	Corner	Centre	Corner	Centre	Corner	Centre	Corner	Centre	Corner	Centre	Corner
L9G	8	46	1	66	0	79	1	58	2	52	5	52
L16R	0	66	0	66	0	47	1	47	3	49	5	31
L16B	1	58	1	78	0	67	1	58	6	61	2	52
L16G	6	55	0	85	3	87	2	76	0	82	6	51
L17R	0	70	1	79	0	66	0	73	8	38	4	40
L17B	8	51	7	57	4	50	1	53	6	45	10	29
L17G	5	31	0	66	6	52	3	58	2	41	3	43
L18R	2	57	0	70	0	55	9	51	2	42	15	42
L18B	11	49	6	55	10	36	5	49	6	43	13	28
L18G	9	50	1	55	4	44	2	70	3	40	5	41

H: High dose group
***Number*:** Cage number
R: Red
B: Blue
G: Green